Bacterial contamination of platelet concentrates

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Molecular Tools and Applications

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BACTERIAL CONTAMINATION OF PLATELET CONCENTRATES

MOLECULAR TOOLS AND APPLICATIONS

ACADEMISCH PROEFSCHRIFT

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geboren te Beni Abdellah, Marokko

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	dr. H.W. Reesink

In memory of my grandfather and my aunt

To my parents and my uncle

Contents

Chapter 1

General Introduction

Introduction

Until the late 1960s blood collected in glass bottles was transfused to recipients as whole blood. The introduction of closed sterile plastic blood bag systems of polyvinylchloride permitted separation of blood into several components and therewith 'blood component therapy'. This therapy includes the use of only the needed blood component for the patients, allowing optimal utilization of donated whole blood. By means of differential centrifugation whole blood can be separated through an integrated system of bags into components. The basic components of blood are red blood cells, platelets and plasma. Red cells are transfused to improve oxygen delivery from the lungs to the cells of the body, platelets are essential in hemostasis. Plasma is transfused to patients who need multiple clotting factors. However, the major part of plasma is not transfused as such but further fractionated into different plasma components (such as clotting factors, immunoglobulins and albumin).

Transfusion of blood components involves many hazards. Among these, transmissions of viral and bacterial infections are the most known. Bacteria in donated blood have already been recognized as a risk for infectious disease when blood was collected and stored in glass bottles [(1)]. The introduction of closed sterile bag systems has contributed to the reduction of this risk. Nevertheless compared to the great decline in the occurrence of transmission of viral diseases in the last decades, septicemia due to blood components contaminated with bacteria, particularly platelets persists. At present it is estimated that 1 in 3,000 platelet concentrates (PCs) may induce sepsis in the recipient, whereas the risk to transmit viral infections is 1 in 200,000 (HBV) to approximately 1 in 2,000,000 (HIV, HCV) [(2,3,4)].

In this introduction first the processing of whole blood into components will be described followed by a description of the methods currently applied to screen PCs for bacterial contamination.

Processing of whole blood into components

Collection of whole blood begins with inserting a needle into a vein of the arm of a healthy volunteer. Prior to this the site of venepuncture is disinfected. The blood flows through the needle into a sterile collection bag containing an anticoagulant. The collection is ended by heat sealing the tubing and thereby closing off the blood bag. Drawn whole blood is used as source for blood component preparation. Upon subjection of this blood to centrifugation the platelets, leukocytes, and red cells fractionate according to their size and density.

By low speed centrifugation a layer of platelet-rich plasma (PRP) containing leukocytes and a layer of red cells mixed with leukocytes and some platelets is formed. The PRP is transferred

to another bag and subjected to a second hard spin. The major part of platelet-poor plasma is then removed and the pellet of platelets is resuspended in about 60 mL of plasma. This PRP can be transfused as single units or can be pooled (4 to 6 units) prior to transfusion [5]. The units can be filtered to reduce the number of leukocytes. Reduction of leukocytes is beneficial in preventing adverse reactions in recipients [6].

The red cells can also be leukocyte-reduced by using a dedicated filter. The platelet-poor plasma is immediately frozen at or below -30°C .

Another method of separating whole blood consists of high speed centrifugation [7,8,9]. Three fractions are obtained: a cell free plasma, a buffy coat fraction containing 90% of the platelets, 70% of the leukocytes and some red cells, and a red cell fraction comprising residual leukocytes and some platelets. These 3 fractions can be separated in different bags. Prior to transfusion the red cells fraction is diluted in an additive nutrient solution. Due to the high hematocrit, red cells are very viscous and do not flow easily through infusion lines. For this reason cell suspensions are diluted. Leukocyte-reduced red cells can be prepared by filtration. The plasma is immediately frozen at or below -30 °C. To obtain a therapeutic dose of platelets for transfusion to an adult, 4 to 6 units of ABO identical buffy coats are pooled together with a unit of plasma or platelet additive solution. Following a soft spin centrifugation the platelet-rich supernatant is transferred through a leukocyte-reduction filter to a platelet storage bag.

Blood components can also be prepared following the apheresis method: this method involves the use of a blood cell separator where blood circulation of the donor is connected to a centrifuge. With this system separation of blood takes place during the collection procedure. The intended component is transferred to a bag, whereas other blood components are returned to the donor.

Storage of blood components

Red cells are stored at +2 to +6 °C. Depending on the nutrients in the storage medium, plasma or additive solution, the storage time can be extended up to 42 days with preservation of quality.

Plasma is freshly frozen to maintain labile clotting factors, and can be stored for up to 2 years at temperatures below -30 °C.

PCs are stored at +20 to +24 °C with continuous gentle agitation for up to 5 days. Under optimal conditions, storage of PCs for up to 7 days without compromising the quality is possible.

In the early days of transfusion, platelets were stored at 4°C [1,3]. Under these conditions their viability and haemostatic activities were affected. Alternatively, storage at room temperature for 3 days was proposed in the 1970s. With the development of gas permeable plastic bags to further preserve the quality of platelets at room temperature in 1981, the shelf life of PCs was extended from 3 to 5 days, and in 1984, it was further prolonged to 7 days. However storage under these conditions was facilitating for growth of bacteria. Due to the increase in the number of cases of platelet contamination with bacteria and instances of transfusion-transmitted bacterial infections, the maximally allowed storage time of 7 days was reduced back to 5 days in 1986.

Nowadays, a storage time for up to 7 days is permitted by the Food and Drug Administration (FDA) in the United States provided that a validated system for screening of PCs for bacterial contamination is implemented. In the Netherlands, bacterial screening of all PCs became mandatory since November 2001. The implementation of such a detection system allowed the prolongation of the storage time of PCs to 7 days [10].

Over the years, the storage time of platelets at room temperature appeared to be correlated to the occurrence of bacterial contamination. This problem was a concern since the introduction of blood transfusion in medical practice. As early as 1951 Borden and Hall reported 2 fatalities linked to the transfusion of whole blood contaminated with bacteria [1]. Since then, numerous cases of platelets contamination and instances of transfusion-transmitted bacterial infections have been published. For example, during the period of 1998 to 2000, six cases of platelet transfusion-related fatalities were reported to FDA[11]. The UK surveillance system, SHOT (Serious Hazards of Transfusion) programme, reported 26 transfusion-transmitted infections, including 6 deaths between 1995 and 2002 [12]. From 1994 to 1998 the French Blood Agency Haemovigilance system has registered 18 deaths caused by transfusion of bacterially contaminated blood components [13,14]. Hence, to exclude this risk, blood centers are recommended to implement a system to assure the absence of bacterial contamination prior to transfusion of PCs.

Screening for bacterial contamination of PCs

Bacteria implicated in contamination

Most organisms detected in contaminated PCs are a variety of Gram-negative and Gram-positive bacteria. The organisms implicated in contamination comprised coagulase-negative *Staphylococcus* spp., *B. cereus*, *Streptococcus* spp., *Peptostreptococcus* spp.,

Propionibacterium acnes, *Klebsiella pneumoniae*, and diphteroids [11,15,16,17,18]. The majority of these organisms belong to the human skin flora. The frequency of contamination with these bacteria varies between countries and between blood centers within the same country. Clinical sequelae due to transfusion of bacterially contaminated platelets range from no symptoms to mild fever to acute sepsis, hypotension and death.

Sources of contamination

Although the source of contamination of PCs is difficult to trace, it is generally believed that contamination can be introduced via blood of the donor, during collection of blood or during the blood component manufacturing process [20,21,22].

Blood donor

An asymptomatic bacteraemia in donors can be responsible for contamination of collected whole blood and subsequent products. Several cases of transfusion associated *Salmonella* sepsis have been reported. A special case of contamination of PCs with *Salmonella enterica* resulting in sepsis in the recipient was described by Jafari et al. [23]. The source of contamination was linked to a pet reptile of the donor. This example emphasizes the importance of careful selection of donors.

Also transient bacteremia in the donor may cause transfusion-associated septicemia in recipients. Endoscopic examination or dental procedures such as tooth extraction might predispose a donor to a transient bacteremia. As a preventive measure, in many countries donors who have been submitted to dental procedures are deferred from donating blood for a few days to one week.

Blood collection

Contamination can be due to inadequate disinfection of the skin at the phlebotomy site. Dependent on the method and materials used to prepare the donor's arm, bacteria from the surface of the skin can be introduced in the blood collection bag. However bacteria from other sources than skin flora may be responsible for contamination as well. For example a fatal sepsis due to transfusion of PCs contaminated with *Clostridium perfringens* was lately reported [24]. This bacterium was cultured from the venepuncture site of the arm of one of the donors. The donor frequently carried his baby in the crook of his arm after changing nappies. It is likely that the skin of the arm of the donor became contaminated by fecal flora in this way. This case highlights the importance of optimal skin cleansing methods.

Contamination may also arise from disinfectants used to prepare the phlebotomy site. Cases of contamination as a result of contaminated disinfectants with *B. cepacia* were reported by Garcia-Erce et al. [25].

It has been postulated that coring of skin during the phlebotomy process may facilitate the entrance of bacteria into the collection bag. To reduce the rate of contamination through this route, several measures have been taken. These include the improvement of methods to disinfect the skin of the donor and the diversion of the first 10 - 40 mL aliquots of whole blood. This latter method appeared promising as described by De Korte et al. [26]. The authors reported a reduction of 0.35% to 0.21% in the prevalence of bacteria after diverting the initial volumes of whole blood. The reduction concerned in most cases bacteria belonging to the skin surface.

In another study McDonald et al. [27] reported a reduction in contamination of 47% when diversion of the initial aliquots was applied. The reduction reached 57% when diversion was done in combination with an improved donor-arm disinfection procedure.

To date, diverting the first aliquots of blood is becoming a routine procedure during collection of whole blood in many blood centers [28].

Blood manufacturing

PCs may be contaminated due to improper manufacturing of blood products or the use of unsterile equipment and devices (due to pinholes or unsterile connections). It is also possible that samples may become contaminated during inoculation of the culture bottles of the BacT/Alert system as part of the quality control (QC) of PCs.

Although rarely, contaminated equipment, such as blood bags used in blood collection, has been implicated as the source of bacterial contamination of PCs [29,30]. To provide safer practices all aspects of blood collection, processing, storing, and transfusion are continuously improved.

Detection methods

Metabolic parameters and visual examination

Bacterial growth typically results in glucose consumption and acid production. This latter causes a decrease of pH in the environment where the bacteria reside. Consequently abnormal glucose concentrations and low pH have been widely used as indicators of the presence of bacteria in PCs. To this end, several glucose devices and pH meters have been employed such

as multireagent strips (dipstick methods). These methods have been evaluated in several studies [31,32,33]. Although these tests are rapid, easy to perform and inexpensive, they are not very sensitive, since they detect only levels of at least 10^7 to 10^8 CFU/mL of bacteria. Moreover, some species of bacteria do not detectably decrease the pH of PCs. Recently, Tarrand and coworkers [34] showed the inability of these reagents to detect *Staphylococcus epidermidis*. The analysis of PCs spiked with 100, 1000 and 10,000 CFU/unit showed no change by the reagent strip method after 5 days of routine storage, while all these units were culture positive. Metabolic parameters, therefore, are not sensitive enough to be used as a screening tool of PCs.

The cessation of platelet swirling has also been used as indicator of bacterial contamination [31]. Swirling is a phenomenon related to the morphology of platelets: fresh platelets are discoid. During storage they may undergo a change in shape from disc to sphere. This morphologic alteration is thought to be accompanied by a loss of viability. The swirling effect occurs as a result of reflection of light scattered by discoid platelets in movement. This scattering is reduced or absent in non-discoid spherical platelets. In addition it is generally believed that bacterial metabolism produces a lower pH leading to a morphology change and decrease of this swirling effect. However, a bacterial load as high as 10^7 - 10^8 CFU/mL is required to induce this process. The swirling can be recorded semiquantitatively as present, absent, or questionably present. Owing to the subjectiveness of interpretation and lack of reproducibility, the observation of the swirling effect is not suited to form the basis for routine testing of PCs for bacterial contamination.

Microscopy

Microscopic examination of PCs has been proposed as a mean of screening PCs for bacterial contamination. Gram stain and Acridine Orange are commonly used [35]. These staining methods are hampered by a low sensitivity, since only high levels of bacterial contamination can be detected. The lower limits of detection of Gram stain and Acridine Orange are 10^5 - 10^6 and 10^4- 10^5 CFU/mL, respectively.

Other microscopic methods include epifluorescence microscopy. This method combines fluorescent nucleic acid staining and automated epifluorescence microscopy. This latter was initially evaluated with PCs contaminated with *Escherichia coli* and *Staphylococcus epidermidis*. The detection limit ranges between 3 to 5 x 10^3 bacteria/mL. The test is

accomplished in about 95 min when analyzing 6 samples. Further validation studies using other bacterial strains are necessary to demonstrate the utility of this method [36].

Other systems contemplating the use of epifluorescence microscopy are under investigation. Among these are the Scansystem (Hemosystem, Marseille, France) [37]. This new approach consists of 3 major steps: first, platelet aggregation is induced by a monoclonal antibody. Second, DNA is labeled with a specific fluorescent marker. Finally the bacteria are concentrated on a membrane surface for enumeration by solid-phase laser scanning cytometer. The cytometer is linked to an epifluorescence microscope. This system was validated with 9 bacterial species. Dependent on the type of the bacterium present a detection limit of 10-10^3 CFU/mL was recently reported [37]. The test is rapid and the results can be obtained in less than 90 min.

DNA/RNA-based methods

Methods using probes targeting ribosomal DNA or RNA of bacteria are widely employed as rapid and sensitive approaches for screening PCs for bacterial contamination.

An assay with Electrochemiluminescence (ECL)-linked universal bacterial RNA probe has been applied. In this assay a specific probe targeting bacterial 16S rRNA, coupled to ECL is used. In a study reported by Rider et al. [38], a sensitivity of approximately 10^5 CFU/mL was obtained. Attempts to enhance the ECL assay sensitivity in a later study conducted by the same authors resulted in the improvement of sensitivity to 10^4 CFU/mL [39]. Nevertheless, this sensitivity is not sufficient to detect low initial levels of bacteria. In addition, the complexity and the duration make this assay difficult to incorporate into routine use.

Pall Bacteria Detection System

The Bacteria Detection System (BDS) system (Pall Corporation, NY) relies on measurement of oxygen consumption as an indicator of bacterial growth [40,41]. In this system 2 to 3 mL PCs sample is filtered to remove platelets and white blood cells and then incubated in a sample pouch for at least 24 hours at 35°C. Thereafter the percentage of oxygen is measured with an oxygen analyzer. An O_2 percentage of less than or equal to 19.5 is used as a cut-off value to differentiate between contaminated and non-contaminated units. According to a pilot study reported by Ortolano et al. it was shown that BDS was able to detect 100 to 500 CFU/mL after 24 hours incubation. However, slow growing bacteria may need longer incubation times to achieve these levels. This system allows the detection of aerobic and facultative anaerobic bacteria. Strict anaerobic microorganisms are not detected. In a recent study reported by Rock

et al, a clinical validation of the BDS system was conducted on 12,062 of random donor platelets. 5 units were tested positive. This result was confirmed by manual methods. The test is easy to perform and requires less than 5 min to be completed. This method is especially useful to be used at the hospital shortly before transfusion.

Immunoassay

Pan Gen Detection (PGD) technology (Verax Biomedical Inc., Worcester, MA) targets the conserved antigens lipopolysaccharide (LPS) and lipoteichoic acid (LTA) which are present on Gram negative and Gram positive bacteria, respectively [(42)]. These antigens are present on the surface of the bacteria and are present in > 200,000 copies/cell. This technology is used in an immunoassay format to detect a wide range of bacteria.

Preliminary studies showed 100% sensitivity and 100% specificity compared to automated culture. The assay is rapid (20 minutes) and is capable to detect approximately 10^3 CFU/mL. Further clinical validation studies are needed to assess the utility of this method.

Automated culture methods

The BacT/Alert (bioMérieux, Durham, NC) culture system is widely implemented to rapidly and feasibly detect microorganisms in blood and body fluids. This fully automated culture system is based on the detection of CO_2 as marker of bacterial growth. For culturing two bottles are used, one for aerobic and the other for anaerobic bacteria. The bottles contain appropriate media to support growth and to ensure optimal CO_2 production. In the bottom of the bottles a CO_2 sensor is present separated from the media by a membrane. CO_2 production by growing bacteria causes a pH decrease. This causes the sensor to change color, which results in an increase of red light reflection [(43)].

The system is easy to use and requires only that a culture bottles be inoculated with 5-10 mL sample of PCs, after which they are loaded into an incubator. These samples are monitored continuously during the incubation (at 35 – 37^{o}C) until a positive signal is recorded or for maximally seven days.

To date, many blood centers (including The Netherlands) have implemented this method as a routine means to screen PCs for bacterial contamination. Several evaluations studies with spiked samples as well as clinical trials have shown the reliability and sensitivity of this system [(44,45,46,48,49,50,56,57)]. In some countries the use of the BacT/Alert allowed the extension of the shelf life of PCs from 5 to 7 days. With the BacT/Alert 1-10 CFU/mL can be detected. Most bacterial species can be detected within 24 to 48 hours. However, slow growing

bacterial species may require more than 48 hours to be detected [54,55]. During this delay, PCs may be issued for transfusion before any contamination has been detected. Possibly the blood product may have been infused to a recipient. Furthermore, during inoculation of the culture bottles, which implies an open system, contamination may occur, and hence, false-positive results. False-negative results have also been reported, due to low initial levels of bacteria [56]. These disadvantages constraint the use of this system for monitoring bacterial contamination of PCs.

Table 1: Currently available detection methods along with their detection limits and times to obtain results.

Detection system	Detection limit (CFU/mL)	Rapidity (min)
Metabolic parameters	10^7 - 10^8	< 5
Swirling	10^7 - 10^8	< 5
Gram/AO stain	10^4 - 10^6	30
Epifluorescence	3 - 5x10^3	95
Scansystem	10^1 -10^3	90
DNA/RNA probes	10^4	?
Pall BDS	10^2	1440
Immunoassay	10^3	20
BacT/Alert culturing	1-10^1	480-1440

In summary, various approaches to detect bacterial contamination prior to transfusion of PCs are possible. Selection of an appropriate method will be based on the combination of the sensitivity and the rapidity of an approach (see Table 1). In addition, it is worthwhile to balance the benefit, risks and costs of each screening method before introduction into routine use.

Pathogen reduction/inactivation

New methodologies aimed at reducing all pathogens (if present) contaminating PCs, are currently under clinical investigations. These methods are based on photochemical inactivation of bacteria and some of these are already available. An example hereof is the Intercept technology [58]. This system uses the synthetic psoralen, amotosalen HCL and illumination with UVA light. Amotosalen intercalates into helices of DNA and RNA, without forming covalent bonds. Upon exposure to UVA light, covalent bonds are formed between the amotosalen and the DNA or RNA that results in the permanent inactivation of the nucleic

acids. The residual amotosalen-HCL and their photodegradation products are removed by absorption onto a special device.

This approach has been tested on apheresis platelets (stored for 5 days) and was shown to be effective in inactivating bacteria. In addition, it was effective in inactivating a range of viruses (eg. HIV-1, HIV-2, HCV, HBV, HTLV-1, HTLV-2, cytomegalovirus, Parvovirus B-19) and parasites (P. falciparum, T. crusi, L. mexicana). This method is attractive, especially for preventing the growth of bacteria.

These preliminary reports are encouraging; however issues of toxicity of residual photochemical agents and the effect of inactivation on the platelet quality have to be investigated [14,59].

Aim of this thesis

Bacterial contamination of PCs with an estimated incidence of 1 in 2000 to 1 in 3000 units is acknowledged to be the most common transfusion-transmitted infections risk. To detect bacterial contamination in PCs prior to transfusion different methods are currently implemented, evaluated or under investigations. As mentioned above, each detection method has limitations and as yet, no method has gained full acceptance. Therefore, developing an 'ideal' detection method is still a challenge for blood centers. This method should have the following characteristics: 1) since initial levels of bacteria that might be present in PCs are thought to be low, in the order of 1 to 10 colony forming units (CFU)/mL, a very sensitive detection method is required. This is especially necessary when the time of testing is closer to the time of collection. Less sensitive method may be used shortly before transfusion. 2) the method should be broadly applicable to all species of bacteria. 3) the method should be highly specific, as false-results would lead to unnecessary wastage of blood. 4) the method should be rapid, allowing detection in a timely manner before issuing of the blood products. Furthermore the method should be easy to perform, requiring a minimum of specialized training and equipment.

Developing an assay that would meet these requirements was the main purpose of this thesis. Based on 5'Nuclease chemistry, a real-time quantitative PCR assay was designed. In this assay a universal probe and primers set was used which specifically detects eubacterial 16S rDNA of the Domain Bacteria.

Initially, the assay was adapted to detect bacterial DNA in PCs. Further optimization resulted in the achievement of a sensitivity of 1 CFU equivalent/PCR. However, the performance of

this broad-range rDNA PCR when directly applied for screening of bacterial contamination in PCs, can be affected by different factors. Among the most important of these is the efficiency of DNA isolation from the PCs and the occurrence of false-positive results caused by the amplification of DNA not present in the actual PCs sample to be analyzed, but present in the reagents used in the PCR. These two issues are addressed in ***Chapter 1***. After solving the problem of contaminating DNA in the PCR reagents and optimizing the DNA extraction method, an internal control was constructed to monitor the efficiency of automated nucleic acid extractions from PCs (**C*hapter 3***). This control relies on the amplification of the generic allelic group of the HLA-DQA1 locus. During a preliminary validation of the assay, false-positive results were generated as a result of contaminating DNA present in the commercial nucleic acid extraction kit reagents. A strategy to circumvent this problem is reported in **C*hapter 4***. Thereafter the clinical validation of the assay was performed as described in **C*hapter 5***. The validation was carried out on routinely prepared PCs, in conjunction with the BacT/Alert culture system as the gold standard. The study described in **C*hapter 6*** was conducted to further determine the optimal sampling time of PCs when using real-time quantitative PCR as a screening tool for bacterial contamination. In **C*hapter 7*** amplified fragment length polymorphism (AFLP) technique is applied to study the molecular relatedness of *Propionibacterium* isolates derived from PCs and red blood cells concentrates (RBCs) from the same donations. This is done in an attempt to identify the source of contamination of blood products with *Propionibacteria*. Finally, a new approach to study the presence and viability of bacteria (*E. coli*) in growth medium and PCs is developed and evaluated in **C*hapter 8***. In this real-time rt-PCR an RNA standard is constructed and used to determine the quantities of DNA and RNA as indicators of the presence and state of viability of *E. coli*. The effect of two antibiotics on the DNA and RNA content is also assessed.

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Chapter 2

Optimization of real-time PCR assay for rapid and sensitive detection of eubacterial 16S ribosomal DNA in platelet concentrates

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ABSTRACT

A real-time PCR assay was developed to allow rapid detection of eubacterial 16S rDNA in platelet concentrates. Two DNA isolation methods, the MagNA Pure LC system and the NucliSens Extraction kit were used to prepare template DNA from platelet concentrates. Both methods proved to have a detection limit of 1 CFU equivalent/PCR. However, contaminating DNA present in the PCR reagents can hamper this high sensitivity. Digestion of all PCR reagents with the restriction enzyme *Sau*3A I prior to PCR amplification proved to be effective to eliminate this contaminating DNA, while the sensitivity of the assay remained unchanged.

This broad-range PCR assay combined with an automated DNA extraction method provides a valuable diagnostic tool for screening of platelet concentrates for bacterial contamination.

INTRODUCTION

Bacterial contamination of blood products is a major cause of transfusion-related morbidity and mortality. Due to their storage at 20-24^{o}C, platelets are responsible for most of the cases of blood component-associated sepsis (1, 7).

The microorganisms most commonly implicated in contamination of platelet concentrates (PCs) belong to the skin flora, among them, *Staphylococci, Streptococci*, *Propionibacterium*, *Bacillus* spp*, Serratia marcescens*, *Micrococcus* spp, and a number of different Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (6).

To demonstrate the presence of bacteria in PCs different methods have been followed (15). In the present study the development of a rapid and sensitive PCR assay based on TaqMan technology to detect bacterial contamination in PCs is described.

To assess the presence of microorganisms in blood and other body fluids real-time PCR has been widely applied. In these assays primers designed to recognize conserved bacterial 16S ribosomal DNA gene sequences are used. Recently, Nadkarni *et al*. (16) has reported the use of a set of universal primers and probe to estimate total bacterial load in clinical samples. Yet, the performance of this broad-range rDNA PCR, when directly applied for screening of bacterial contamination in PCs, can be affected by different factors. Among the most important of these is the efficiency of DNA isolation from the PCs and the occurrence of false-positive results caused by the amplification of DNA present in the PCR reagents.

In the study reported in this paper, two different isolation methods were used to prepare template DNA from PCs: a fully automated method with the MagNA Pure LC instrument and a manually extraction procedure with the NucliSens Extraction kit.

The MagNA Pure is a benchtop instrument (10), which offers a standardized, rapid and reproducible method for DNA isolation. During purification, the risk of cross-contamination is substantially reduced. Together, these characteristics make this system appealing for use in routine diagnostic laboratories.

NucliSens extraction, which is based on the Boom purification method (2, 19), can be conducted either fully automated (with a NucliSens Extractor) or manually. The difference between both procedures resides in the replacement of the centrifugation step to separate silica bound nucleic acid in the manual method by filtration through a silica filter by air pressure in the automated method.

A major problem associated with the use of universal PCR-based bacterial detection systems is the occurrence of false-positive results caused by the amplification of contaminating DNA (present in PCR reagents). Several authors have reported on contamination of PCR reagents with genomic DNA from microorganisms used in the preparation of enzymes, such as *Taq* polymerase (3, 4, 5, 9).

Attempts to reduce the amount of contaminating DNA in real-time PCR have been described previously (12, 14, 20):

Kox *et al*. (11) introduced as first the use of uracil-N-glycosylase (UNG) with dUTP instead of dTTP to prevent false-positive results due to amplicon carryover contamination. Other strategies included the use of 8-methoxypsoralen and long-wave UV light (13), DNase treatment (8, 9), incubation with different restriction enzymes (4, 5, 18), use of reagents minimally contaminated with DNA (16). Finally, Yang *et al*. recently reported on ultrafiltration of the PCR mix (21). None of these methods, however, proved very effective, especially when low copy numbers of bacterial rDNA have to be discriminated from contaminating DNA in the PCR reagents. In addition, the majority of these strategies also affect the sensitivity of the real-time PCR. Here two strategies to reduce contaminating DNA in real-time PCR amplification are evaluated and found suited to meet the requirement of the PCR system to detect bacteria in PCs without affecting the high sensitivity of the assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions:

For this study *Escherichia coli* was used as a prototype. *E. coli* (ATCC25922) was grown in Luria-Bertani broth at 37^{o}C to match a turbidity of 0.5 Mc Farland (1.5 x 10^{8} CFU/mL). Serial dilutions (10^{-1} to 10^{-6}) were made in phosphate-buffered saline (PBS); the suspensions were plated on blood agar plates to determine the number of CFU/mL.

Preparation of platelet concentrates (PCs):

After collection, whole blood is stored for 16-20 hours at room temperature before preparation of components. Briefly, with a hard-spin centrifugation step, whole blood is separated into plasma, buffy coat (containing leukocytes and platelets) and red cells. The buffy coat is used as the source of platelet concentrates (17).

After separation five buffy coats and one unit of plasma are pooled and centrifuged (soft-spin). The resulting platelet-containing supernatant is filtered (to remove residual leukocytes)

before transfer to a storage bag. The PCs are stored at 20-24^{o}C with continuous gentle agitation for up to 7 days.

Spiking assay: 1 mL of PCs was spiked with 100 µL of serial dilutions of *E. coli*. The number of bacteria added to the PCs ranged from 10 to 20,000 CFU/mL.
Spiking of whole blood (WB), red cell concentrates (RCC) and plasma (PL) was performed as described for PCs.

DNA extraction:
MagNA Pure procedure: In this method MagNA Pure Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, The Netherlands) was used and the purification performed as specified by the manufacturer. DNA was extracted from a 200 µL aliquot of spiked material. The DNA was eluted in a final volume of 50 µL. With this protocol 32 samples can be extracted within 90 minutes.
NucliSens procedure: The purification was carried out with the NucliSens Extraction manual kit (bioMérieux, Boxtel, The Netherlands) according to the manufacturer's instruction. DNA was purified from a 2000 µL aliquot of spiked PCs. The extracted nucleic acid was eluted in a final volume of 50 µL (19). This procedure can also be performed in a fully automated way with the NucliSens Extractor. The capacity of this instrument, however, is only 10 samples per run with a processing time of approximately 45 minutes.
DNA was also extracted from serial dilutions of a pure culture of *E. coli* following the same MagNA Pure extraction procedure as described above.

Real-time PCR assay:
The PCR was set up to amplify a highly conserved region of bacterial 16S ribosomal DNA using the forward primer 5'-TCCTACGGGAGGCAGCAGT-3', the reverse primer 5'-GGACTACCAGGGTATCTAATCCGTT-3'and the probe (6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'-(TAMRA). This universal primers and probe set was designed by Nadkarni et al. (16) to amplify a fragment of 466 bp.
The PCR reactions were performed in a total volume of 25 µL using the TaqMan Universal PCR Master Mix (containing 1 x TaqMan Universal PCR Master Mix, AmpliTaq Gold DNA Polymerase, AmpErase UNG, dTPs with dUTP, Passive Reference 1 and optimized buffer components, Applied Biosystems, Foster city, CA USA). The reactions comprised 900 nM of

each of the universal forward and reverse primers and 200 nM of the fluorescent labeled probe and 5 µL of template DNA. Negative controls (unspiked PCs) were included throughout the procedure. No template controls (NTC) with water instead of template DNA were incorporated in each run.

The PCR reactions were performed under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The PCR assay was conducted on the ABI 7700 Sequence Detection System (PE Applied Biosystems). Analysis of data was done with the SDS software (version 1.7) supplied by the manufacturer.

Pre-treatment of the PCR mixture to reduce contaminating DNA:

Restriction endonuclease digestion with Sau3A I: Prior to the addition of template DNA, the PCR mixture was subjected to digestion with the enzyme *Sau*3A I (1 Unit per PCR reaction, New England Biolabs, Beverly, MA USA). After incubation at 37°C for 30 minutes, the solution was heat inactivated at 65°C during 20 minutes. PCR reactions were subsequently performed as described above. The size of the PCR product was verified on 1.5% agarose gel stained with ethidium bromide.

Ultrafiltration: Before the addition of template DNA, the PCR mixture was filtered with an Amicon Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, Mass. USA) as described by Yang et al. The PCR mix was passed through the YM-100 filter unit at 100 x *g* for 30 minutes.

DNase I treatment: DNase I (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was added to the PCR mix (0.2 U/PCR reaction) without template DNA. The solution was incubated at 37°C for 10 minutes, followed by heat denaturation at 65°C for 15 minutes. After this treatment template DNA was added and the PCR was carried out.

RESULTS

Detection limit of the real-time PCR assay:

To determine the sensitivity of the assay, PCs spiked with serial dilutions of *E. coli* were employed. The result of each PCR reaction is indicated by a threshold cycle (C_T) value. The C_T value is defined as a measure of the fluorescence generated by cleavage of probe against a fixed base line threshold and is proportional to the amount of the PCR product. The software of the TaqMan system automatically calculates the C_T values. As shown in Table 1 the detection limit of the assay was 1 CFU equivalent/PCR reaction. This corresponds to 100

CFU equivalent/mL spiked PCs when the MagNA Pure kit is used, and to 10 CFU equivalent/ml when the DNA extraction is performed with the NucliSens Extraction kit. This difference is due to the starting volume of the clinical sample used to extract DNA. Based on the size of *E. coli*, which is 4639 kb, 1 CFU is equivalent to approximately 5 fg DNA.

Reduction of contaminating DNA:

Routine precautions to prevent contamination of the PCR mixture were always in use (e.g., physical separation of the different steps of the PCR assay). Because the initial PCR assays showed a high background, as can be deduced from the threshold value of the NTC (Table 1), probably due to the presence of traces of bacterial DNA in the enzymes used in the PCR, the PCR mixtures were pre-treated in different ways to reduce this source of contamination. The initial attempts included predigestion of the PCR mixture with *Alu*1/*Rsa*1, withdrawal of AmpErase uracil N-glycosylase from the Universal PCR Master Mix, prefiltration of the mixture using different columns and membranes. All these strategies were unsuccessful and did not result in the removal of contaminating DNA (data not shown).

Digestion of the PCR mixture with *Sau*3A I reduced the amplification signal of the NTC. As shown in Table 2, the C_T value increased with two PCR cycles. The C_T values of the reaction mixtures containing template DNA increased accordingly with 1-2 PCR cycles. This, however, did not affect the sensitivity of the assay, since 1 CFU equivalent/reaction was still detectable.

Table 1. Detection limit of real-time PCR assay performed with DNA isolated from PCs (spiked with*E. coli*) using either MagNA Pure or NucliSens extraction procedure.

PC spiked with *E. coli* (CFU equivalent/PCR)	DNA extraction method	C_T value[a]
8×10^1	NucliSens	27.84 ± 0.43
8×10^0	"	31.58 ± 0.13
4×10^0	"	32.80 ± 0.21
2×10^0	"	33.40 ± 0.26
1×10^0	"	34.73 ± 0.70
2×10^2	MagNa Pure	31.11 ± 0.42
4×10^1	"	33.42 ± 0.10
4×10^0	"	34.83 ± 0.83
1×10^0	"	35.42 ± 0.45

[a] Mean ± standard deviation of three independent experiments.

Table 2. Detection limit assay with DNA extracted from a pure culture of *E. coli* and serially diluted[a].

Sample type	C_T for template *E. coli DNA* in the following units (CFU equivalent/PCR)[b]							
	NTC	7.5×10^5	7.5×10^4	7.5×10^3	7.5×10^2	7.5×10^1	7.5×10^0	10^0
Untreated	34.56±0.08	21.39±0.40	25.73 0.24	29.9±0.21	33.37±0.7	34.8±0.27	35.07±1.04	34.58±0.34
+ *Sau3*A1	37.46±0.09	22.62±1.32	25.83±0.01	30.2±0.06	33.77±1.69	35.97±1.4	36.61±2.39	36.03±2.80
+ Filter	40.00±0	22.15±0.03	26.11±0.81	30.51±0.03	34.59±0.11	37.6±0.08	40.00±0	40.00±0
+ DNase I	24.53±0.08	26.20±0.78	21.95±1.03	23.47±0.20	23.89±0.55	24.8±0.02	24.25±0.2	25.04±0.06

[a] DNA extraction was done with the MagNA Pure LC instrument. The results of different pretreatments (digestion with Sau3AI, filtration, and treatment with DNase I) are also presented.
[b] Mean ± standard deviation of three independent runs.

Filtration of the PCR mixture through a YM-100 filtration unit resulted in an increase of the C_T value of the NTC to 40, which indicated that by this step contaminating DNA was eliminated. Filtration of the PCR mixture before addition of template DNA, however, also reduced the sensitivity of the assay (Table 2).

Pretreatment of the PCR reaction mixtures with DNase I led to an unexpected decrease in the C_T values of all PCR reactions, including that of the NTC (table 2).

Similar results were also found when PCR assays were performed with DNA isolated from spiked PCs (table 3).

Decontamination experiments were also conducted in other blood components, i.e. plasma (PL), red cell concentrates (RCC) and whole blood (WB) to find out if the effectivity of the pre-treatment was affected by the type of clinical material used. In these assays employment of DNase I was omitted. The results are presented in figure 1A, B and C, respectively.

Digestion with Sau3A I caused an increase of 1-3 PCR cycles in all tested blood components, whereas ultrafiltration with YM-100 resulted in an increase of 1-7 PCR cycles.

Treatment with the restriction enzyme allowed the detection of 3 CFU equivalent/reaction in PL and RCC, and 1.5 CFU equivalent/reaction in WB. When prefiltration was applied the sensitivity of the assay was reduced to 30 CFU equivalents/reaction in both PL and RCC, and to 15 CFU equivalents/reaction in WB.

DISCUSSION

Over the last decade, broad-range real-time PCR has been increasingly used for the detection of pathogens in molecular diagnostics. To apply this approach to detect bacterial contamination in PCs, two critical factors have to be taken into consideration: the DNA must

be extracted as efficiently as possible from the PCs, and the PCR mixture must not be contaminated by DNA, that is not present in the PCs to be tested. A poor efficiency of DNA extraction may restrict the sensitivity of the assay, while DNA that is contaminating PCR reagents can serve as a template in PCR reactions, producing false positive results.

In this study two isolation methods have been used to prepare template DNA from PCs: a fully automated method with the MagNA Pure LC instrument, and a manual method with the NucliSens isolation kit. The PCR performed on DNA obtained by either of both methods was very sensitive and as little as 1 CFU equivalent/reaction mixture could be detected. DNA extraction with the NucliSens method, however, requires a 10-fold larger volume of PCs than with the MagNA Pure, 2000 µL instead of 200 µL.

The problem of contamination of the PCR mixture by DNA, not present in the actual PCs sample to be analyzed, but present in the reagents used in the PCR, was solved by two different methods: restriction endonuclease digestion with *Sau*3A I or removal of DNA by ultrafiltration. The aim was to get rid of any contaminating DNA that may be present in the reagents to be used in the PCR assay, before the addition of the template DNA, extracted from the clinical material. Removal of any trace of DNA from the reaction mixtures allows the achievement of a maximal sensitivity of the real-time PCR assay.

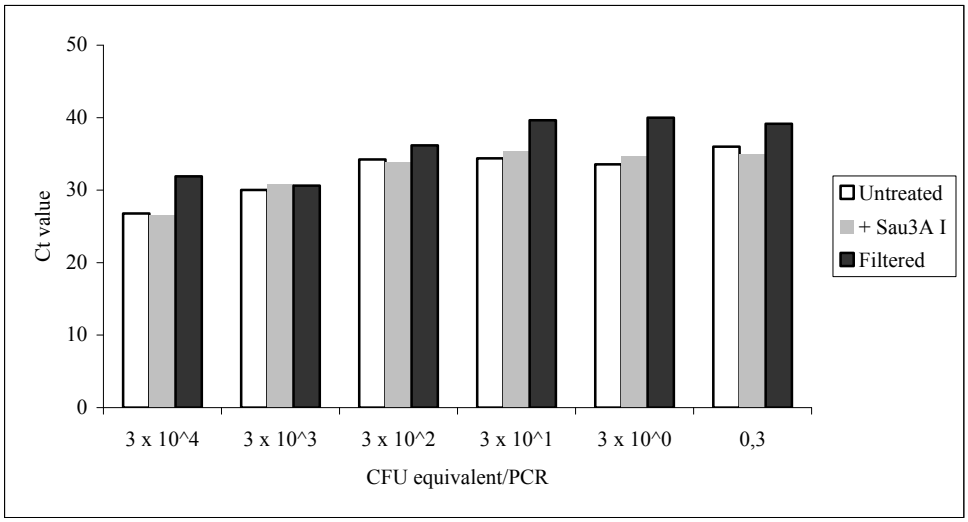
Digestion with *Sau*3A I proved to be effective in reducing contamination of the PCR mixture, as shown by the increase of the mean C_T of the NTC. The higher the C_T value of the template control, the broader the range of the C_T values that can be achieved by the PCR reactions

Table 3. Detection limit of the assay with DNA extracted from PCs spiked with *E. coli* DNA without treatment and pretreated by *Sau3*A I, ultrafiltration and DNase I.

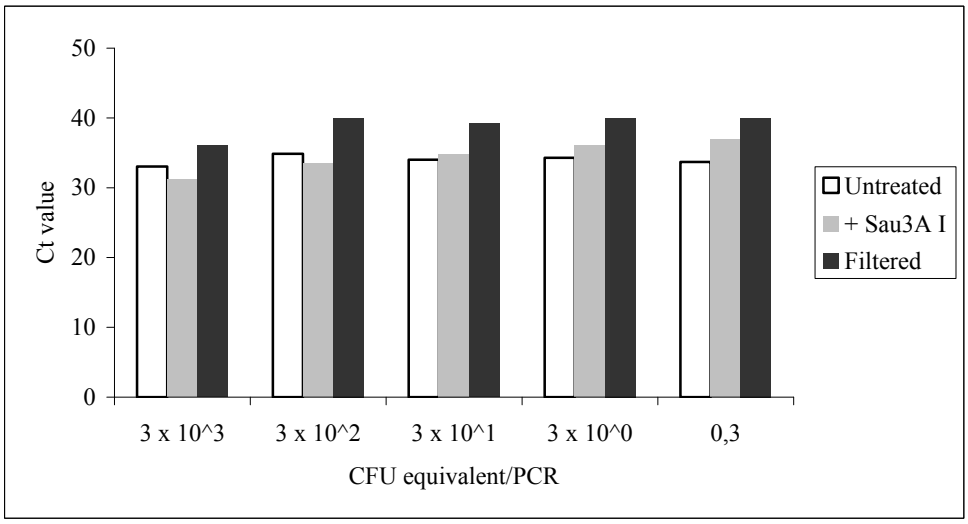
Sample type	C_T for template DNA isolated from PCs spiked with *E. coli* in the following amounts (CFU equivalent/PCR)[A]				
	NTC	3×10^3	3×10^2	3×10^1	3×10^0
Untreated	34.56 ± 0.08	29.59 ± 1.26	33.40 ± 0.31	34.93 ± 0.66	34.72 ± 0.30
+ Sau3A1	37.46 ± 0.09	29.82 ± 1.68	33.8 ± 0.36	35.84 ± 0.43	36.01 ± 0.74
+ Filter	40.00 ± 0	30.55 ± 0.05	34.01 ± 1.11	39.37 ± 0.40	40.00 ± 0
+ DNase I	24.53 ± 0.08	23.63 ± 0.72	23.00 ± 0.36	23.15 ± 1.08	25.40 ± 0.43

[a] C_T (threshold cycle) values are the mean of 3 independent runs. NTC: no template control.

A



B



C

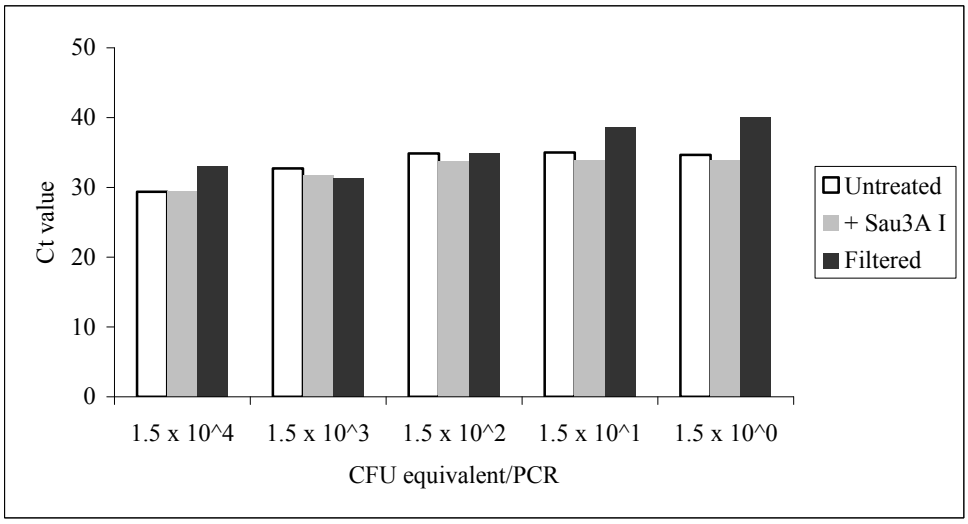


Figure 1. Detection limit of the assay with *E. coli* DNA detected in plasma (A), red cell concentrates (B) and whole blood (C) with or without treatment. Template DNA was isolated from 1 mL plasma, red cell concentrates or whole blood spiked with *E.coli* in the amounts (CFU equivalent/PCR) plotted on the *x*-axis. The extraction of DNA was performed with the MagNA Pure LC instrument. The C_T values for each reaction are represented on the *y*-axis.The extraction methods differ also in the operating time: with the MagNA Pure LC instrument results can be obtained within 4 hours, while the NucliSens Extraction kit requires 6 hours.

containing template DNA, and hence the better the sensitivity. In the case of pre-treatment with *Sau3*A I, the detection limit of this already very sensitive assay remained 1 CFU equivalent/PCR reaction (5 fg DNA).

Following the prefiltration procedure a mean C_T value of 40 was generated for the NTC. However the sensitivity of the assay performed with DNA isolated from spiked PCs was reduced to 30 CFU equivalents/PCR reaction. Thus, *Sau3A I* seems to be effective especially when detection of only a few molecules of a target DNA sequence is required. When a detection limit of fewer than 10 CFU equivalent/reaction is not needed, prefiltration of the PCR mixture seems to be more appropriate.

Both methods can be simply integrated in the PCR procedure to overcome the problem of contaminating DNA. Pre-treatment with *Sau*3A I extends the assay by 50 minutes, whereas the prefiltration step prolongs the PCR with 30 minutes. Advantages and disadvantages of each strategy should be therefore taken into account when selecting the most convenient method.

Pre-treatment with DNase I resulted in an unexplainable decrease in the C_T values of all PCR reactions. This nonspecific endonuclease cleaves both DNA strands. During single-strand digestions, DNase I causes gaps and nicks that can be repaired by DNA polymerase. Possibly, this process makes the target sequence more accessible and the amplification of contaminating DNA more efficient. As a result a decrease in C_T values of all PCR reactions occurred. Since the results of this pre-treatment were not consistent, the use of this enzyme to reduce contaminating DNA is not favorable.

To prevent transfusion-associated bacterial sepsis, rapid and sensitive methods are needed for pretransfusion screening of PCs. Real-time PCR assay in combination with the automated MagNA Pure DNA extraction method meets these requirements: the assay takes only 4 hours to be completed and has the capability to detect very small numbers of bacteria.

When contaminating DNA in the PCR mixture forms a limitation, digestion with *Sau3*A I can be used to solve this problem while preserving the sensitivity of the assay.

Before this assay can be implemented as a tool to screen PCs for bacterial contamination, further validation studies are needed. Currently the performance of this PCR to detect the bacterial species most frequently found in PCs is being evaluated and compared with current automated culturing methods.

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Preface to chapter 3

Real-Time PCR assays are prone to inhibition by many compounds that might be present in clinical materials. Inhibition may cause the generation of false-negative results. To avoid false-negative and false-positive results, controls should be incorporated in nucleic acid amplification assays. Controls serve to assess the integrity of each step of the PCR assay (i.e. extraction and amplification steps).

Since extraction of nucleic acid is the first step in the 16S rDNA PCR, a suitable and reliable control is indispensable to monitor the efficiency of this procedure. The aim of the following chapter was essentially to develop such an isolation control. A highly conserved region of the generic allelic group of HLA-DQA1 locus was selected and used as an endogenous control sequence. During the validation of the PCR assay with this target, it was shown that the assay was also suitable to be used as a method to estimate the number of residual white blood cells (WBCs) in WBC-reduced PCs. Reduction of WBCs in blood products is routinely carried out in blood centers to reduce the adverse effects of transfusion associated with WBCs. To determine the utility of this method, the performance of this real-time PCR with the set of primers and probe designed as internal control, was compared with a method (current gold standard) used in most blood centers to count the amount of residual WBCs in PCs.

Thus, the assay was shown to have two applications: (i) as extraction control, and (ii) high-throughput and sensitive assay for monitoring residual WBCs in PCs (as will be described in the following chapter).

Chapter 3

Real-Time amplification of HLA-DQA1 for counting residual white blood cells in filtered platelet concentrates

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ABSTRACT

BACKGROUND: A real-time PCR assay based on amplification of the generic allelic group of the HLA-DQA1 locus was developed and validated to assess its suitability in quantitating low levels of white blood cells (WBCs) in filtered platelet concentrates (PCs).

STUDY DESIGN AND METHODS: The performance of this assay was characterized by assessing its lower limit of detection, accuracy and reproducibility. To determine the detection limit serial dilutions of non-filtered PCs with known quantities of WBCs were prepared. DNA was isolated with the MagNA Pure LC Instrument. The real-time PCR was performed with a set of primers and probe targeting a highly conserved region of the well-evaluated HLA-DQA1 gene. To determine the analytical sensitivity and accuracy of the assay WBC concentrations ranging from 300 to 0.03/ μL were tested with real-time PCR and flow cytometry. In addition, 126 random PCs were investigated to assess the capacity of the PCR method to quantify residual WBCs in clinical specimens. The intra- and inter-assay variations were also evaluated.

RESULTS: A sensitivity of 0.2 white blood cell equivalent/μL (1.5×10^4 white blood cells equivalent/unit) was achieved. The assay was shown to be accurate and the HLA-DQA1 gene was reproducibly and consistently amplified in all tested samples (CV less than 5%). Overall, the results of the PCR assay correlated well with those of the flow cytometry. Using the PCR assay, a concentration of 3 WBC/μL (corresponding to approximately 1×10^6 WBC per unit) was detected with 100% accuracy.

CONCLUSION: Real-time PCR is rapid, sensitive, accurate and reproducible. Hence this approach may prove suitable in routine monitoring of residual WBCs in PCs.

INTRODUCTION

Reduction of white blood cells (WBCs) in cellular blood components is nowadays common practice in blood centers. This reduction is beneficial to prevent a variety of transfusion complications, including febrile reactions, HLA alloimmunization, immunomodulation and transmission of viruses incorporated in leukocytes (1).

To monitor WBC-reduction efficiency different studies have been performed to count residual WBCs in WBC-reduced red cell concentrates and platelet concentrates (2, 3). Microscopy, flow cytometry and volumetric capillary cytometry are the most commonly used methods (4, 5, 6, 7). Although these methods are suitable for counting residual WBCs in blood components, high throughput, sensitive and robust methods are desirable.

As recently shown by Lee *et al.* (8) amplification of a highly conserved region of the *HLA-DQα* gene may be used as a potential strategy to measure residual WBCs in blood components.

In this study a rapid and specific real-time amplification assay of the generic allelic group of HLA-DQA1 locus to estimate residual WBCs in filtered platelet concentrates (PCs) is described. To investigate the suitability of this approach, for the monitoring of residual WBCs in filtered PCs the performance of the PCR assay was characterized and compared to flow cytometry.

MATERIALS AND METHODS

Preparation of WBC-reduced platelet concentrates

PCs were prepared using the buffy coat method. In brief, 500 mL whole blood was collected from healthy volunteers in triple top-and-bottom bag systems (Composelect T2988, Fresenius Hemocare, Emmer-Compascuum, The Netherlands) with citrate phosphate-dextrose (CPD) as anticoagulant, and saline-adenine-glucose-mannitol (SAGM) as additive solution for red cells. After collection, whole blood is cooled to 20-24 ^{o}C with butane-1,4-diol containing cooling elements. After storage during 16-24 hours at this temperature, blood is separated in cell free plasma, buffy coat and red cells by applying high-speed centrifugation. The platelet concentrates are then isolated from the buffy coat layer by low speed centrifugation.

Five ABO-identical buffy coats are pooled with one unit of plasma in an integrated system consisting of pooling bag, a leukoreduction filter (ImugardIII-PL4P filter, Terumo, Japan) and a platelet storage bag. During process control of leukoreduced blood components, residual WBCs are counted with conventional methods such as flow cytometry. The number of

residual WBCs is determined to verify if they meet the standard, which is defined as less than $1x10^6$ WBCs/unit in more than 90% of the units (9).

DNA extraction

DNA extraction was performed in a fully automated system with the MagNA Pure LC instrument (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions. Two hundred µL of each sample was purified with the MagNA Pure Total Nucleic Acid Isolation Kit. The nucleic acid was eluted in 50 µL elution buffer. Amplification was performed with a real-time PCR assay. With the MagNA Pure LC system 32 nucleic acid extractions can be performed in less than 90 minutes.

Primers and probe design

Primers were designed to amplify a fragment of a highly conserved region of the HLA-DQA1 gene. This 216 bp fragment spanned the second exon of all currently known alleles at the HLA-DQA1 locus. The sequences of the
alleles were downloaded from the website: http/www.anthonynolan.com/HIG. (10).
The primer pair consisted of the forward primer 5'-TTGTACCAGTTTTACGGTCCC-3', the reverse primer 5'-TGGTAGCAGCGGTAGAGTTG-3', the probe was (VIC)-5'-TTCTACGTGGACCTGGAGAGGAAGGAG-3'-(TAMRA).
PCR amplification reactions were performed in a final volume of 25 µL with the TaqMan Universal PCR Master Mix (1x TaqMan Universal PCR Master Mix, AmpliTaq Gold DNA Polymerase, AmpErase UNG, dTPs with dUTP, Passive Reference 1 and optimized buffer components, Applied Biosystems, Foster city, CA USA). The reaction mixtures consisted of 900 nM of each of the forward and reverse primers and 200 nM of the fluorescent labeled probe and 5 µL of template DNA. Reaction mixtures with water instead of template DNA were incorporated in each run as negative controls (no template controls: NTC). The PCR conditions were: 50°C for 2 minutes and 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.
The PCR assay was conducted on the ABI 7000 Sequence Detection System (PE Applied Biosystems). Analysis of data was done with the SDS software supplied by the manufacturer.
Amplification, detection and analysis of the results were completed within 2 hours.

Assessment of performance of the assay

The performance of the assay was assessed by determination of the following characteristics: detection limit, accuracy and reproducibility.

Detection limit: to determine the lower limit of detection serial dilutions of non-filtered PCs were prepared. The number of WBCs in these PCs was automatically determined with Sysmex K1000 (TOA, Tokyo, Japan). In the serial dilution experiments double-filtered PCs were used as diluent.

Accuracy and reproducibility: to determine the accuracy of the PCR assay, its analytical performance was compared with that of flow cytometry (as reference method). To accomplish this, experiments were carried out with samples containing WBCs concentrations ranging from 300 to 0.03/μL. These samples were prepared from a tube of citrated whole blood. After collection the number of WBCs in this tube was automatically determined with Sysmex K1000. Subsequently the whole blood was serially diluted in PBS to obtain final concentrations of 300, 30, 3, 0.3 and 0.03 WBCs/μL. For each dilution six aliquots were prepared and counted in duplicate by both PCR and flow cytometry.

When flow cytometry was applied the number of WBCs in the samples was measured with the LeucoCOUNT kit (BD Biosciences, San Jose, CA, USA). This kit included TruCOUNT tubes with a lyophilized pellet with a fixed number of fluorescent counting beads. An aliquot of 100 μL was added to these tubes including 400μL of LeucoCOUNT reagent. This reagent consisted of RNase, a detergent to make the cells permeable for Propidium Iodide (PI) and PI for DNA staining. The samples were incubated in the dark at room temperature for 5 minutes. Thereafter measurements were performed on a FACSCalibur flow cytometry (10 000 events were counted for each sample) according to the instructions of the manufacturer.

To evaluate the capacity of the PCR method to quantify residual WBCs in PCs, the assay was applied to 126 samples of routinely filtered (WBC-reduced) PCs. DNA was extracted from these samples and amplified in the real-time PCR. The threshold cycle (C_T) values were determined and coefficient of variance (CV) calculated. The C_T value is the cycle number at which the fluorescence generated by cleavage of probe exceeds a fixed base line threshold.

To further characterize the assay both intra- and inter-assay variations were studied. Intra-assay variations were tested by extracting DNA from 20-fold replicates of a sample of WBC-reduced PC followed by analysis with real-time PCR. To assess inter-assay variations DNA was extracted from replicates of five samples of WBC-reduced PCs and subjected to PCR amplification in 5 independent runs. The mean, SD, and CV% were determined.

N bRESULTS

To determine the lower limit of detection of the PCR assay, DNA isolated from serial dilutions of non-filtered PCs with a known quantity of WBCs (5 x 10^5 to 5 x 10^0 WBCs/mL) was analyzed by real-time PCR. As depicted in figure 1 the assay was linear between 10^3 and 10^0 WBC equivalent/PCR reaction indicating that 1 WBC equivalent/PCR reaction is the lower limit of detection. This is equivalent to 0.2 WBCs/µL (50 WBCs equivalent/mL).

The analytical accuracy of the PCR method in estimating low numbers of WBCs was assessed by comparing the results of the serial dilutions with the expected values. The serial dilutions were made in PBS to exclude any contamination with WBCs that could possibly be present in the double-filtered PCs leading to higher estimation than expected from the dilution itself. The results were also compared with those obtained by flow cytometry. As shown in Table 1 the PCR values showed high agreement with the expected values. Sufficient correlation was also achieved with the flow cytometry. Both methods were able to detect as low as 0.3 WBCs/µL. The extent to which the observed (measured) results span the expected results is given in Table 2. The 95% CI of the observed results was calculated as well. As defined in the guidelines of the Biomedical Excellence for Safer Transfusion (the BEST) accuracy was considered to be satisfactory when 80% of the observed results were within 20% of the expected values (5).

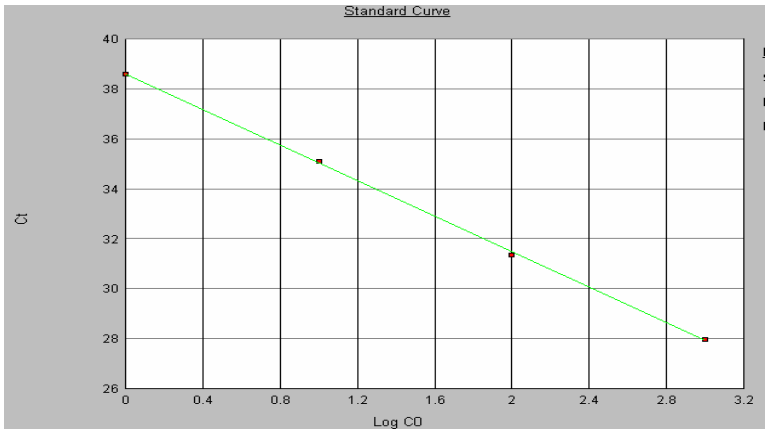

Figure 1: DNA isolated from non-filtered PCs representing 10^3, 10^2, 10^1 and 10^0 WBCs/PCR reaction were used to determine the detection limit of the HLA-DQA1 PCR assay. Slope: -3,534, Intercept: 38,583, R^2: 0,999. The X-axis of the graph represents the starting copy number of the HLA-DQA1 gene expressed as log WBCs equivalents/PCR reaction. The Y-axis represents the threshold cycle (C_T).

Table 1. Number of WBCs estimated with flow cytometry and HLA-DQA1 PCR in samples with known concentrations*.

Expected number of WBC/μL	Observed WBC count/μL			
	Flow cytometry		HLA-DQA PCR	
	mean ± SD	CV(%)	mean ± SD	CV(%)
300	258 ± 13.4	5.2	300.96 ± 13.06	4.3
30	25.3 ± 1.37	5.40	30.35 ± 5.1	17
3	2.0 ± 0.3	15	3.28 ± 0.25	7.6
0.3	0.2 ± 0.03	15	0.21 ± 0.04	19
0.03	undetectable		undetectable	

* Results are the mean of six experiments.

Table 2. Accuracy of flow cytometric method and the real-time PCR assay.

Expected number WBCs/μL	Accuracy		95% CI of observed values	
	Flow cytometry	PCR	Flow cytometry	PCR
300	100%	100%	247.5-268.5	290.7-311.2
30	100%	100%	24.2-26.4	26.4-34.3
3	40%	100%	1.8-2.2	3.1-3.5
0.3	70%	70%	0.18-0.22	0.18-0.24

At concentrations above 3 WBCs/μL the expected values of all samples tested with PCR fell within the 95% CI of the observed values. In contrast, the flow cytometry demonstrated an underestimation of the expected value in all cases. Hence, the accuracy of the PCR assay was considered to be better than the flow cytometry.

At concentrations below 3 WBCs/μL both methods showed reduced accuracy. Only 70% of samples gave a result similar to the expected value ± 20%.

Of 126 samples of WBC-reduced PCs the value of residual WBCs ranged between 10-100 WBCs equivalents/PCR reaction (which is equivalent to 2-20 WBCs/μL), the C_T values

varied between 33.71 and 34.65. For these samples the flow cytometry yielded values varying between 0.1 and 0.2 WBC/µL.

The results of the assessment of reproducibility demonstrated a consistent and reproducible amplification of the HLA-DQA1 fragment. In these experiments the number of WBCs ranged again between 10 - 100 WBCs equivalents/PCR. The CV values for both intra- and inter-assay variations were observed to be less than 5% (data not shown).

DISCUSSION

The amplification of the generic allelic group of HLA-DQA1 locus by real-time PCR proved suitable for quantitation of residual WBCs in PCs. A sensitivity of 0.2 WBC equivalent/µL was achieved. This corresponds to 1.5 x 10^4 WBCs equivalents/unit of 300 mL filtered PCs (prepared form 5 donors), which is approximately 100-fold below the maximum tolerated level of WBCs in PCs (1 x 10^6 WBCs/unit) (9). When performing the serial dilutions experiments all measured values corresponded well with the expected values. In general accuracy was greater for the PCR assay than for the flow cytometry. Discordance between observed and expected values was obtained only at concentrations of 0.3 WBC/µL. Overall, the findings demonstrated a good accuracy and reproducibility of the PCR assay.

Although a different set of primers and probe was used, the findings of the study reported here support results obtained by Lee *et al*. (8). In a previous investigation Lee e*t al*. described the use of a high-throughput real-time kinetic PCR (kPCR) assay as an approach to assess the number of residual WBCs in WBC-reduced whole blood. With the PCR test used in that study, the authors could detect 2.4 x 10^3 to 2.4 x 10^4 WBCs per unit of WBC-reduced whole blood. This kPCR, however, was combined with a manual method to prepare template DNA from clinical specimens. Manual extraction procedures are not very attractive when a standardized extraction for all tested samples is required.

Another important difference between the assay described in this report and the assay developed by Lee *et al*. resides in the absence of a specific probe to detect amplified products in the test as developed by them. In addition they used Ethidium Bromide for detection and subsequent quantitation. This could make the distinction between specific and nonspecific amplification difficult.

Other counting methods such as flow cytometry and microscopy enable enumeration of 1 and 0.1 WBC/µL, respectively (4, 5, 6). However, they require expensive instruments and are

labour-intensive. Besides, the performance of microscopy-based methods is influenced by the skills and interpretation of the operator.

With the assay reported in this study a high-throughput of samples can be reached in a relatively short period of time: the automated extraction method is completed within 90 minutes when processing 32 samples and the amplification and analysis take less than 2 hours. Moreover, no pre-treatments (e.g. dilutions) are required prior to counting. The MagNA Pure extraction system in combination with real-time PCR detection offers a standardized, rapid and reproducible assay suited for routine monitoring of residual WBCs in PCs.

Furthermore the generic (universal) nature of the primer and the probe set developed in this study makes it amenable to be used as an internal control to monitor efficiency of automated DNA extraction from blood samples in routine diagnostics laboratories.

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Chapter 4

Removal of contaminating DNA from commercial nucleic acid extraction kit reagents

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ABSTRACT

Due to contamination of DNA extraction reagents, false-positive results can occur when applying broad-range real-time PCR based on bacterial 16S rDNA. Filtration of the nucleic acid extraction kit reagents with GenElute Maxiprep binding columns was effective in removing this reagent-derived contaminating DNA while the sensitivity of the assay was maintained.

A broad-range PCR assay was recently developed, based on real-time PCR technology, to monitor bacterial contamination in platelet concentrates (PCs) (Mohammadi et al., 2003). The assay is rapid and enables the detection of 1 CFU equivalent/PCR. The assay combines the automated MagNA Pure DNA extraction and real-time amplification of a conserved sequence of the eubacterial 16S rRNA gene. The availability of fully automated extraction systems offers the benefit of standardization, high efficiency of purification and reduction of the potential risk of cross-contamination. However, the performance of the assay is compromised by a batch-dependent DNA contamination of the DNA isolation kit reagents (Peters et al., 2004). To achieve optimal sensitivity and reproducibility it is important to remove this contaminating DNA. In the present study a method to eliminate contaminating DNA in MagNA Pure Total Nucleic Acid kit reagents is described.

The real-time PCR assay was performed on 20 samples of PCs (derived from pools of 5 individual blood donors each). The presence of bacteria in these PCs was assessed by culture of a sample in the BacT/Alert (bioMérieux, The Netherlands). By this automated culture system the PCs were deemed to be negative meaning that no bacteria were present.

DNA was extracted from 200 µL of each sample with the commercially available MagNA Pure Total Nucleic Acid Extraction kit (Roche Diagnostics). Amplification and detection were performed simultaneously with the real-time PCR on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR were performed in a final volume of 25 µL with the TaqMan Universal PCR Master Mix (1 x TaqMan Universal PCR Master Mix, AmpliTaq Gold DNA Polymerase, AmpErase UNG, dTPs with dUTP, Passive Reference 1 and optimized buffer components; Applied Biosystems, Foster city, CA USA). The reaction mixtures included 900 nM of each of the universal primers (forward primer 5'-TCCTACGGGAGGCAGCAGT-3', reverse primer 5'-GGACTACCAGGGTATCTAATCCTGTT-3'), 200 nM of the probe (6-FAM-5'-CGTATTACCGCGGCTGCTGGCAC-3'-TAMRA) and 5 µL template DNA. In each assay,

isolation and PCR inhibition controls were incorporated. A negative control (no template control (NTC)) with water instead of template DNA was also included. Amplification conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were evaluated based on the threshold cycle (C_T) values of each PCR reaction.

Unexpectedly, bacterial DNA was detected in all tested samples (including NTCs): C_T values ranging from 33 to 34 cycles were obtained.

Subsequently, the products of the PCR were purified and subjected to automated sequencing with the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequencing was conducted with the same universal primers. Sequence analysis by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) revealed sequence similarities with bacteria inhabiting water and moist soil environments. Among these were *Burkholderia* spp, *Pseudomonas saccharophilia, Ralstonia* spp, *Alcaligenes* spp, and other related bacteria.

Since contaminating DNA in the PCR reagents was removed by digestion with *Sau*3AI (Mohammadi et al., 2003) and all measures to prevent cross-contamination were taken throughout the whole procedure from processing, DNA isolation to PCR amplification and detection, evidence was accumulating for a link between the MagNA Pure isolation reagents and the occurrence of false-positive results in the PCR assay.

Three approaches to remove contaminating DNA were evaluated and their effect on the sensitivity of the PCR was assessed.

First, the MagNA Pure Total Nucleic Acid kit reagents (Wash Buffer I, Wash Buffer II, Wash Buffer III, Lysis/Binding Buffer and Elution Buffer of the extraction kit) were sonicated (Sanyo Soniprep 150). By sonication DNA fragments were generated. Due to their minute size these pieces of DNA could not be amplified by PCR. Only target DNA from genuine contamination of PCs would be then amplified and detected. The duration of sonication was varied between 2 min and 30 min. This, however, did not sufficiently reduce the positive signals derived from the contaminating DNA (data not shown).

In a second attempt to eliminate contaminating DNA, a probe was designed which targeted a sequence of the 16S ribosomal RNA gene that is homologous in all *Burkholderia* spp. and related contaminants identified by sequencing. The sequence of the probe was (VIC)-5'-TAAGCACCGGCTAACTACGTGCCAGC-3'-(TAMRA). This probe was used in combination with the universal primers and probe set in a competitive real-time PCR. As the

C_T values of the PCR reactions were almost equal when both probes were used, it was difficult to establish a clear difference between the targets. This strategy was therefore not pursued.

A third method consisted of filtration of the reagents of the Total Nucleic Acid kit with the aid of the GenElute Plasmid Maxiprep binding columns (Sigma-Aldrich Chemie Gmbh, Germany). These silica-based membrane columns are constituents of a kit for the isolation of plasmid DNA from bacterial cultures. Except Magnetic Glass Particles and Proteinase K, all reagents of the MagNA Pure Total Nucleic Acid Extraction kit were transferred to the columns and centrifuged at 5,000 x *g* for 5 minutes. The flow-through was used in the MagNA Pure system to extract DNA from PCs. Compared to the C_T values obtained with the real-time PCR on DNA extracted with untreated reagents, the C_T values of DNA samples extracted with the treated reagents were higher (Table 1). This demonstrated the reduction of contaminating DNA. The background signals that were still obtained after filtration are attributable to the presence of contaminating bacterial DNA in the PCR reagents. This signal became completely negative (undetectable) after digestion of the PCR mixture with *Sau*3AI (Table 2). As described in a previous study (Mohammadi et al., 2003) this pretreatment was carried out routinely to remove contaminating DNA in the PCR reagents.

The MagNA Pure LC Microbiology kit (Roche Diagnostics) recently became commercially available, after which the manufacturer of the MagNA Pure reagents was notified of the problem of DNA contamination. All the reagents of this new kit are M^{GRADE} quality, ensuring that they are tested for and free of bacterial and fungal DNA contamination.

Table 1. Amplification of 16S rDNA performed with DNA isolated from five separate PCs using the indicated MagNA Pure extraction kits.

Extraction kit	PCs samples[a]					
	1	2	3	4	5	NTC
Total Nucleic Acid	34.59±0.30	35.32± 0.37	34.57± 0.28	35.58± 0.63	34.52± 0.22	34.77± 0.70
Total Nucleic Acid + filtration	37.30± 0.5	37.3± 0.21	37.58± 0.60	37.73± 0.77	37.07± 1.61	37.13± 0.11
M^{GRADE}	37.7± 0.63	36.47± 0.54	36.28± 0.48	37.11± 0.35	37.24± 0.60	38.17 ±1.07

[a] The results are expressed as C_T values. The mean ± standard deviation of three independent runs is given.
NTC: no template control.

To confirm the purity of the reagents of this kit, DNA was isolated from the same samples as described above and the results were compared to the results obtained with the untreated- and the column purified reagents. As shown in Table 1, the C_T values generated with DNA extracted with untreated reagents augmented substantially when the extraction from the same samples was conducted with the M^{GRADE} kit. Comparable results were also found when the reagents of the Total Nucleic Acid kit were first filtered with the columns. The included controls indicate the efficacy of DNA extraction and the absence of inhibitors of amplification. These results indicate that the use of commercial M^{GRADE} reagents or filtration of the reagents from the conventional MagNA Pure Total Nucleic Acid Extraction kit are equally effective methods to avoid contamination of PCR by DNA present in the extraction reagents.

To assess the effect of filtration on the sensitivity of the assay, PCs were spiked with bacterial suspensions to reach a final amount of 10^3, 10^1 and 10^0 CFU equivalent/PCR. The sensitivity of the assay performed with reagents that had been passed over the column was not altered: 1 CFU equivalent/PCR was still detectable (Table 2).

The use of GenElute columns was also effective for the elimination of contaminating DNA in QIAamp manual DNA extraction kits. Performance of PCR with DNA isolated from PCs without any pretreatment of the extraction reagents showed a mean C_T value of 36.84 ± 0.9 (3 independent experiments) although contaminating DNA in the PCR reagents was removed. When reagents of QIAmp DNA Blood Mini kit reagents (Qiagen) were filtered with the columns prior to their use for the isolation of DNA from PCs (negative by culture), no amplification products were observed (mean C_T 39.48 ± 0.4) indicating that they were free from contaminating DNA. A complete absence of amplification signals was observed when

Table 2. C_T values determined by real-time PCR for PCs samples extracted with Total Nucleic Acid kit, Total Nucleic Acid kit + filtration and M^{GRADE} kit.

Extraction kit	NTC	PCs samples[a]	PCs spiked with *E. coli* (No. CFU equivalent/PCR)[b]		
			$1x10^3$	$1x10^1$	$1x10^0$
Total Nucleic Acid	33.84 (38.93)	34.91 (37.71)	23.74 (23.16)	30.24 (30.01)	32.41 (32.25)
Total Nucleic Acid + filtration	39.28 (und)[c]	37.39 (und)	23.50 (21.41)	31.70 (28.72)	34.96 (34.34)
M^{GRADE}	37.48 (und)	36.96 (und)	24.80 (22.59)	32.22 (31.31)	36.77(36.32)

[a] Culture-negative specimen was used as a negative control.
[b] Real-time PCR results are expressed as C_T values. Template DNA was isolated from PCs using Total Nucleic Acid kit, Total Nucleic Acid kit + filtration and M^{GRADE} kit. The effect of filtration on the sensitivity of the assay was assessed by amplifying DNA extracted from PCs spiked with different amounts of *E. coli* suspensions. Numbers in parentheses are the C_T values obtained after digestion of the PCR mixture with *Sau*3AI. This pre-treatment was carried out to remove contaminating DNA in the PCR reagents.
[c] No amplification signal was detectable.

both filtration of the extraction reagents and digestion of the PCR reagents with *Sau*3AI were carried out. This method may therefore also be employed to eliminate DNA from reagents in other diagnostic PCR-based assays with manual DNA extraction methods.

Newly developed, high throughput standardized automated extraction methods that substantially improve conventional manual methods are becoming increasingly popular in clinical laboratories. However, it is of utmost importance to consider potential pitfalls of these methods especially those concerning of false-positive results due to reagent contamination. Van der Zee *et al*. (van der Zee and Crielaard, 2002) and Evans *et al*. (2003) have previously demonstrated the contamination of Qiagen DNA extraction kits with *Legionella* DNA.
Hence, DNA contamination should be taken into account when applying both automated and manual extraction methods in routine clinical microbiology.

Contamination derived from extraction reagents can result from a number of sources. In this study the identified bacteria are inhabitants of a variety of aquatic environments. Other authors have reported before that these bacteria are indigenous to ultrapure water in industrial systems (Kulakov et al., 2002; Taghavi et al., 1996). Therefore, it is likely that this problem originates from the distilling systems of the manufacturer.

The method introduced here to overcome the problem of contaminating DNA in the MagNA Pure Total Nucleic Acid kit reagents, is easy-to-perform and rapid. There is no significant loss of volume after filtration of the reagents. The use of Genelute columns may also prove useful for the filtration of other MagNA Pure kits reagents for example the Large Volume Total Nucleic Acid kit. In addition it is less expensive to use the columns compared to the M^{GRADE} kit at present. Moreover, the M^{GRADE} kit is only suited for the processing of 100 µL sample volumes whereas the Total Nucleic Acid kit and the Large Volume Total Nucleic Acid kit are designed for DNA extraction from samples of 200 µL and 1000 µL respectively. Finally, the results indicate that purification of the reagents with the columns did not hamper the sensitivity of the PCR assay.

In conclusion, purification of reagents to be used in DNA-based assays by centrifugation over GenElute columns is a generally applicable, easy and fast procedure that can be incorporated in large number of protocols, especially in case of possible false-positive results. It can be then decided to use the GenElute columns to purify the reagents from contaminating DNA. For this pretreatment, modification of optimized procedures and protocols are not necessary.

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Chapter 5

Detection of bacteria in platelet concentrates: comparison of broad-range real-time 16S rDNA PCR and automated culturing

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ABSTRACT

Background: Up to now several methods have been employed to monitor the presence of bacteria in platelet concentrates (PCs). Based on real-time PCR technology, a broad-range 16S rDNA assay was previously developed. In the present study, the assay was validated and its performance compared to that of an automated culture system to determine its usefulness for rapid routine screening of PCs.

Study design and methods: The assay was applied to PCs derived from pools of 5 individual blood donors each. The presence of bacteria in these PCs was routinely assessed in a BacT/Alert automated culturing system. Cultures were maintained until a positive signal was detected or for up to seven days when remaining negative. The PCR assay was performed with DNA extracted from the same samples as used for culturing. DNA extraction was done with the MagNA Pure automated extraction system. PCR amplification was performed with a set of universal primes and probe targeting eubacterial 16S rDNA.

Results: A total of 2,146 PCs were tested. Eighteen (0.83%) samples were found to be contaminated. These samples were positive by both methods. All contaminants were identified as bacteria belonging to the common human skin flora. These included *Propionibacterium* spp. (n = 7), *Staphylococcus* spp. (n = 6), *Bacillus* spp. (n = 2), *micrococcus* spp. (n = 2), and *Peptostreptococcus* spp. (n = 1). All microorganisms were detected within 3 days, except *Propionibacterium* spp. and *Peptostreptococcus* spp. that required longer (3 to 5 days) anaerobic culturing to be detectable by BacT/Alert. By real-time PCR these contaminants were detected within a C_T range of 24.4-36.9. Estimation of the bacterial load in PCs by real-time PCR showed that the initial levels of contamination varied between 13.6 and $9x10^4$ CFU equivalents/PCR reaction. Taking into consideration the nature and growth characteristics of bacteria present in the PCs on one hand and the fact that real-time PCR detect DNA from both viable and nonviable bacteria on the other hand a correlation between a high initial bacterial load and a decreased time to detection by BacT/Alert was calculated.

Conclusions: This study showed that, compared to culture in the BacT/Alert system, the PCR assay had a sensitivity of 100% and a specificity of 100%. This real-time PCR assay has a much shorter turnaround time of four hours, which offers the possibility to test and obtain results on PCs before release or the day they are transfused. This would permit the withdrawal of contaminated PCs before transfusion.

INTRODUCTION

Transfusion of platelet concentrates (PCs) contaminated with bacteria may cause serious septic reactions that can be fatal to the recipient. Prevention or reduction of the occurrence of these septic reactions is therefore a major challenge faced in blood banking and transfusion medicine (1, 2). Up to now various techniques, like pretransfusion bacterial screening of PCs, have been adopted by blood centers to reduce the risk of transfusion-associated septic reactions (3, 4, 5, 6). These techniques vary from manual and visual methods to automated blood culture systems (7, 8, 9, 10, 11, 12). Many of these methods have proven to be useful for identification of bacterially contaminated PCs but have also proven to be inconvenient because of the time they require to indicate the presence of bacteria, their low specificity, and their lack of sensitivity when the initial levels of contaminating bacteria are low.

To be useful as an optimal tool for routine screening of PCs, a dependable assay is desirable. The assay should be simple, sensitive enough to detect all clinically significant levels of bacteria, highly specific and rapid to allow the release of PCs for clinical use within hours (5, 13). The aim of the present study was a test that would meet all these criteria. As reported in a previous study (14), a broad-range 16S rDNA PCR assay, was developed and optimized, based on real-time PCR technology. In brief, in this assay the 5'nuclease activity of DNA polymerase is exploited to cleave a dual-labeled DNA probe after hybridization to its target during the elongation step of the annealed primers. After cleavage a fluorescent signal is generated that allows real-time detection of the amplified DNA (15, 16). As described before (14) this very rapid 16S rDNA-targeted real-time PCR has an analytical sensitivity of 1 colony forming unit (CFU) equivalent/PCR reaction in PCs. To assess the applicability of this method for routine use in blood centers, the PCR assay was compared with an automated blood culture system. Routinely produced PCs were therefore screened by both methods.

MATERIALS AND METHODS

Processing of PCs and screening for bacterial contamination

Leukoreduced PCs were routinely prepared following the buffy coat method. Briefly, after collection, whole blood was stored for 16-20 hours at room temperature before preparation of components. With a hard-spin centrifugation step, whole blood was separated into plasma, buffy coat (containing leukocytes and platelets) and red cells. The buffy coat was used as the source of platelet concentrates (17). After separation five ABO-identical buffy coats and one unit of plasma were pooled and centrifuged (soft-spin). The resulting platelet-containing

supernatant was filtered to remove residual leukocytes before transfer to a storage bag. The PCs are stored at 20-24^{o}C with continuous agitation for up to 7 days.

The presence of bacteria in these PCs was routinely assessed in the BacT/Alert automated culture system (bioMérieux, Durham, NC). A sample was obtained within 2 hours after preparation of PCs via a sample pouch integrated in the storage bag. Under aseptic conditions, standard aerobic and anaerobic BacT/Alert bottles were inoculated with 5-10 mL aliquots of PCs from the sample pouch through the integral needle. The samples were cultured until a positive signal was detected or for up to seven days if they remained negative. Positive culture bottles were subcultured for confirmation and identification of microorganisms (6).

During the sampling procedure, duplicate samples were drawn, as used for inoculation of the BacT/Alert bottles, for the PCR assay. These aliquots were frozen at -70^{o}C until further processing.

Nucleic acid extraction from PCs

DNA was isolated from aliquots of PCs using the MagNA Pure LC automated extraction system (Roche Diagnostics, Almere, The Netherlands). Prior to DNA extraction, the reagents of the MagNA Pure Total Nucleic Acid kit were filtered with the GenElute Maxiprep columns (Sigma-Aldrich Chemie Gmbh, Germany). This pretreatment is necessary to eliminate contaminating bacterial DNA present in the reagents of the extraction kit (personal communication). Two hundred µL aliquots of PCs were then loaded in the MagNA Pure and extracted according to the manufacturer's instructions. Extracted nucleic acid was eluted in 50µL elution buffer.

Real-time PCR amplification and detection

A 466-bp fragment of the bacterial 16S rDNA was amplified with a broad-range probe and primers set designed and described previously by Nadkarni et al. (18). The sequence of the forward primer and the reverse primer were 5'-TCCTACGGGAGGCAGCAGT-3' and 5'-GGACTACCAGGGTATCTAATCCTGTT-3' respectively. The fluorescent probe had the following sequence: (6-FAM)-5'-CGTATTACCGCGGCTGCTGGCAC-3'(TAMRA). The PCR reactions were performed in a total volume of 25 µL comprising the TaqMan Universal PCR Master Mix (containing 1 x TaqMan Universal PCR Master Mix, AmpliTaq Gold DNA Polymerase, AmpErase UNG, dTPs with dUTP, Passive Reference 1 and optimized buffer components, Applied Biosystems, Foster city, CA USA), 900 nM of each of the forward and the reverse primers, and 300 nM of the probe and 5 µL of template DNA.

The PCR mixture was subjected to digestion with the enzyme *Sau3*AI prior to the addition of template DNA. Also this pretreatment is necessary to eliminate possible traces of bacterial DNA present in the enzymes used in the PCR (14).

No-template controls (NTC) with sterile water instead of template DNA were included in each run. The amplification was carried out under the following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 min and 60°C for 1 min. The PCR assay was conducted on the ABI 7000 Sequence Detection System (Applied Biosystems). Analysis of data was done with the SDS software (version 1.0) supplied by the manufacturer.

Controls

The efficacy of DNA extraction was assessed by coamplification of the human *HLA-DQA* gene in all reactions (19). The sequence of the forward primer was 5-TTGTACCAGTTTTACGGTCCC-3' and the reverse primer 5'-TGGTAGCAGCGGTAGAGTTG-3'. For the detection of the 216-bp amplicon a probe with the following sequence 5'-TTCTACGTGGACCTGGAGAGGAAGGAG-3' was designed. This probe was labeled on the 5'end with VIC, and with TAMRA on the 3'end.

To ensure that there was no PCR inhibition, control DNA from *Bordetella avium* DNA (5 µL, equivalent to 50 CFU) was added to the PCR reactions with template DNA. The extraction and inhibition controls were performed in the same reaction in parallel with the reactions performed with DNA extracted from the PCs aliquots. In addition negative isolation controls with water instead of PCs and negative reagent controls (NTC) without template DNA were incorporated throughout the whole procedure of extraction and amplification.

Standard calibration curve for quantification

Staphylococcus aureus (ATCC 25923) DNA was used to generate a calibration curve. DNA was purified from tenfold-dilution series of bacterial suspensions with known amounts of CFU. The extraction was conducted following the same procedure as described above. A calibration curve was incorporated in each PCR run and was used for determination of bacterial load in contaminated PCs.

RESULTS

Comparison between real-time PCR and BacT/Alert:

A total of 2,146 PCs was sampled and assessed for the presence of bacteria by both the PCR assay and the BacT/Alert culturing system. After PCR amplification of DNA isolated from

PCs a threshold cycle (C_T) value was generated for each reaction. A C_T value is defined as the PCR cycle number at which fluorescence generated by cleavage of probe exceeds a fixed baseline threshold level. A high C_T value corresponds to a low input of target DNA. The C_T values were used to interpret the results of the PCR assay.

First, a cut-off value for positivity was determined to differentiate between contaminated and non-contaminated PCs. Based on the controls and the analytical validation of the assay a result was considered positive when the C_T value was less than 37 (corresponding to approximately 1 CFU equivalent/PCR reaction). C_T values above this cycle number were considered negative. This interpretation of the results was valid only when DNA extraction was efficient and no PCR inhibition was observed. This implied that the HLA-DQA gene was consistently amplified (C_T range 33-34) and that the amplification of the *B. avium* DNA added to the PCR reactions with template DNA was not inhibited (a C_T range between 33.63 and 34.99 was required). If HLA-DQA1 was not amplified or the PCR appeared inhibited a new aliquot of the same sample of PCs was re-extracted and re-amplified. With regard to the BacT/Alert results, a culture was deemed as positive when a microorganism was identified in the culture bottles.

Eighteen samples were identified as positive by both real-time PCR and BacT/Alert. All specimens that remained negative in the BacT/Alert system were also negative in the PCR assay. Therefore, there was perfect agreement between BacT/Alert and PCR assay.

Estimation of the bacterial load in contaminated PCs:

From the dilution series of the *S. aureus* suspension a standard curve was generated. This curve was used to estimate the bacterial load (reflected as CFU equivalent/PCR reaction) in the PCs. An example of a representative standard curve is depicted in Fig.1. The curve was linear between 10^5 and 10^0 CFU/PCR reaction. By extrapolation of C_T values of the PCs samples absolute quantities were calculated. A summary of the identified bacteria, time to detection of each microorganism by the BacT/Alert, and estimation of bacterial load is presented in Table 1. The majority of the microorganisms identified in the contaminated PCs were Gram-positive bacteria belonging to the common human skin flora. *Propionibacterium* spp. (n = 7) were most frequently isolated, followed by *Staphylococcus* spp. (n = 6). Dependent upon the microorganism involved, the

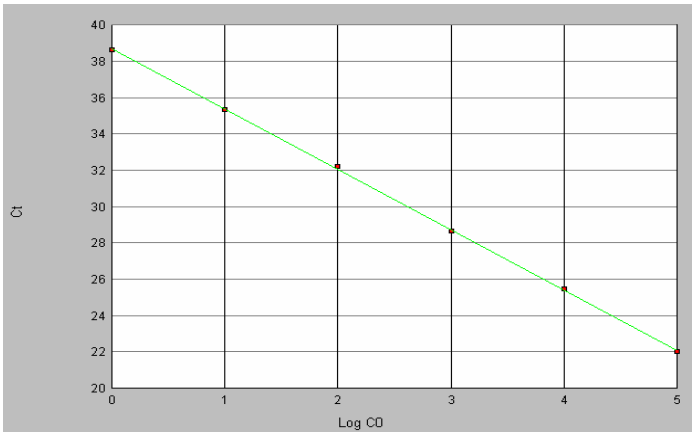

Figure 1. Standard curve generated with DNA extracted from serial dilutions of a pure culture of *S. aureus*. The assay was linear over a wide range varying between 1 x 10^0 and 1 x 10^5 CFU equivalents/PCR reaction. Slope: -3.326, Intercept: 38.704 and R^2: 0.999. The X-axis represents the starting copy number expressed as log CFU equivalents/PCR reaction. Threshold cycle (C_T) values are plotted on the Y-axis.

bacterial load varied between 15.4x10^0 and 1x10^5 CFU equivalent/PCR. Although a relatively high initial amount of CFU was estimated by the real-time PCR, the BacT/Alert required 4 to 6 days to detect the presence of *Propionibacterium* spp. The fastest detection times were recorded for *S. epidermidis* (n = 2): 23.5 to 2x10^2 CFU equivalent/PCR reaction were detected within 2 days.

Micrococcus spp. (n = 2) were detected in the BacT/Alert in approximately 3 days, the bacterial load was 13.6 and 30 CFU equivalent/PCR. *Bacillus* spp. (n = 2) was detected after approximately 5 days of culturing in the BacT/Alert. The presence of *Peptostreptococcus* was detected in one case after 6 days of anaerobic culturing. For the sample contaminated with this bacterium a bacterial load of 2x10^4 CFU equivalent/PCR was determined.

Table 1. Estimation of bacterial load by real-time PCR and time to detection of PCs signaled positive by BacT/Alert

		BacT/Alert		Real-time PCR		
				Bacterial load		
Sample	Bacterium	Time to detection (hrs)	Detection*	Ct	CFU/PCR	CFU/mL
1	*Propoinibacterium* spp.	42	N	24.4	10^5	$5x10^6$
2	*Propionibacterium* spp.	46.8	N	36.7	3.5	175
3	*Propionibacterium* spp.	57.6	N	27.9	$7.1x10^4$	$3.6x10^6$
4	*Propionibacterium* spp.	61.2	N	32.7	10^2	$5x10^3$
5	*Propionibacterium* spp.	62.4	N	26.7	$9x10^4$	$4.5x10^6$
6	*Propionibacterium* spp.	63.6	N	36.2	3.8	$1.9x10^2$
7	*Propionibacterium* spp.	70.8	N	35.8	17.4	$8.7x10^2$
8	S. epidermidis	18.5	A/21[b] N	35.8	23.5	1.2×10^2
9	S. epidermidis	19.2	A/24.8 N	35.9	41.9	2.1×10^3
10	*S. epidermidis*	20.2	A	33.9	$2x10^2$	10^4
12	*S. epidermidis*	40.8	N	35.2	43.6	$2.2x10^3$
11	*S. schleiferi*	34.8	A	36.5	15.4	$7.7x10^2$
13	*S. capitis*	49	N	34.6	325.2	$1.6x10^4$
14	*Bacillus* spp.	52.8	A	34.1	38.2	$1.9x10^2$
15	*Bacillus* spp.	58.8	A	35.2	23.7	$1.2x10^2$
16	*Micrococcus luteus*	37	A	36.9	13.6	$6.8x10^2$
17	*Micrococcus* spp.	42.3	A	36.6	30.08	$1.5x10^2$
18	*Peptostreptococcus* spp.	62.4	N	26.3	$2x10^4$	10^6

* N = detected in the anaerobic culturing bottle of BacT/Alert (N); A = detected in the aerobic culturing bottle of BacT/Alert .

[b] These bacteria were also detected in the anaerobic bottles at the indicated time.

DISCUSSION

Currently, automated culturing is considered to be the gold standard in most blood centers to assess the presence of bacteria in PCs. Although this method is sensitive, it requires up to 48 hours to yield (positive) results for most microorganisms. While the culture is still ongoing, PCs might already be issued (as negative to date) to the hospital and be administered to a

recipient. In addition, a large sample of 5-10 mL is required to inoculate both culturing bottles of the BacT/Alert. This large inoculum is lost from the blood product. In view of these disadvantages an alternative, faster method to test PCs for bacterial contamination was desirable. A 16S rDNA-targeted real-time PCR was previously developed and optimized. In the present study the performance of this assay was compared to that of automated culture. The results of the real-time PCR were in agreement with those obtained with the BacT/Alert. The PCR assay showed the same sensitivity and specificity as culture, with the advantage of a much shorter turn around time. In addition, the PCR permitted an estimation of the number of CFU present in the PCs samples that were tested.

The microorganisms that were detected as contaminants in PCs were: *Propionibacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Micrococcus* spp. and *Peptostreptococcus spp*. Various studies have reported these bacteria to be the common contaminants found in PCs (20, 21, 22, 23). In the current validation study the majority of these bacteria were detected by culture within 3 days, except most *Propionibacterium* spp., which needed 4 to 6 days to be detected in the anaerobic culture bottles of the BacT/Alert. This longer time was needed, despite the high bacterial load that was present in the starting culture (estimated by real-time PCR). It is known that *Propionibacterium* spp. have a prolonged lag phase. Consequently, the bacterium requires long time to proliferate before it becomes detectable with the BacT/Alert.

Although the 16S rDNA-targeted real-time PCR was designed as a sensitive and rapid alternative to blood culture to detect contaminated PCs on a qualitative basis, its real time character provided the opportunity to examine the relationship between number of bacteria present in contaminated PCs and time to detection in the BacT/Alert. A high initial concentration of bacteria did not invariably correlate with a short detection time in the BacT/Alert. Two factors may explain this finding. First, the real-time PCR detects DNA from both viable and nonviable bacteria, while the BacT/Alert is based on detection of CO_2 produced by growing bacteria. Second, since the PCR was performed on samples taken on day 1 of storage of PCs (day 0 is day of collection), it is likely that only few microorganisms were recovered in the sample incubated in the BacT/Alert. Dependent on the growth kinetics of the bacterium present the time needed for detection with the BacT/Alert can vary.
On the other hand, quantitation results determined by real-time PCR support the results obtained by others. Studying bacterial surveillance of platelet concentrates Blajchman *et al*. (24) concluded that all true positive could be detected in units sampled on day 3. Brecher *et*

al. (25) have reported on the low detection efficiency of the BacT/Alert when bacteria are present in numbers below 10 CFU/mL. Hence, it was suggested that 2 to 3 days incubation is needed to allow bacteria to proliferate before reliable detection can occur. In other studies it was observed that all bacteria could be detected within 3 days when starting concentrations of 10 to 100 CFU/mL were used (26).

In summary, the real-time PCR assay, in combination with the automated MagNA Pure nucleic acid extraction system provides a standardized, rapid and sensitive method to monitor bacterial contamination of PCs. The assay offers various benefits above most commonly used systems. Among these the use of closed-tube chemistries, which reduces the risk of contamination of samples during the assay. In addition, with this assay it is possible to process a large number of specimens (96-well format can be used) in a timely manner. The results can be obtained within 4 hours. This is especially favorable when 100% testing of PCs is mandatory.

The short turnaround time of this assay will reduce the risk of transfusion of bacterially contaminated units: PCs can be tested before release or the day they are transfused. To determine the most convenient time for sampling additional evaluation studies of the PCR assay in conjunction with the BacT/Alert are in progress.

The PCR assay does not depend on the growth kinetics of bacteria. This feature permits the extension of the storage time of PCs from 5 to 7 days without compromising the risk of transfusing contaminated units.

However, this broad-range 16S rDNA PCR assay could become a source of false-positive results if no sufficient measures to prevent contamination are taken. These include the use of separate rooms for sampling of PCs, for nucleic acid extraction and for setting up PCR reactions. Other sources of contamination with bacterial DNA like PCR enzymes and extraction reagents must also be precluded. In addition, the use of dedicated devices and disposables needed during the whole procedure of extraction, amplification and detection may greatly increase the costs of the assay. Accordingly, before implementing such an assay as a routine screening tool in a blood center it might be recommended to conduct a cost-benefit analysis.

In conclusion, real-time PCR proved a rapid and reliable method to test PCs for prevention of transfusion of contaminated units.

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Chapter 6

Optimal sampling time after preparation of platelet concentrates for detection of bacterial contamination by quantitative Real-Time PCR

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ABSTRACT

Background and Objectives: A universal quantitative Real-Time (RT) PCR, based on bacterial 16S rDNA to detect bacterial contamination of platelet concentrates (PCs), was developed previously and compared to automated culturing. In the present study, this RT-PCR method was evaluated to determine the optimal sampling time for screening of bacterial contamination in PCs.

Materials and Methods: Routinely prepared PCs were spiked with suspensions of *Escherichia coli*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Propionibacterium acnes* to 1, 10 and 100 CFU/mL and stored at room temperature for 7 days. The presence of bacteria in these PCs was monitored by quantitative Real-Time PCR. As reference method (additional control) BacT/Alert automated culturing was used. For PCR 1 mL aliquots were drawn from all (spiked) PCs on day 0, 1, 2, 3, 6 and 7 of storage. As a control, triplicate samples (10 mL) were inoculated in aerobic and anaerobic BacT/Alert culture bottles immediately after spiking (day 0) and after storage of 1, 2, 3, 6 or 7 days.

Results: With quantitative Real-Time PCR all tested bacteria species were reproducibly detected on day one after inoculation at original concentrations of 10 and 100 CFU/mL. Initial concentrations of 1 CFU/mL were also detected on day 1 except for *E. coli* which was detected only in 1 of the 3 samples and *P. aeruginosa* which was not performed on day 1.

With the reference method bacteria were detected in culture bottles (inoculated on day 0) within a mean time of 20.1 hours, with exception of *P. acnes* which was detected in a mean time of 102.3 and 49.3 hours (for 10 and 100 CFU/mL respectively).

Conclusions: PCR enables rapid detection of low initial numbers of bacteria in PCs. For reliable detection, our results support that sampling of PCs for RT-PCR screening should not be carried out earlier than one day after preparation (48 h after blood collection). Importantly, the RT-PCR approach has the potential to be used before release of PCs from the blood centre or shortly before they are transfused in the hospital.

INTRODUCTION

Screening platelet concentrates (PCs) for bacterial contamination by means of automated culturing is becoming a routine procedure in many blood centres over the world. Studies evaluating the performance of the BacT/Alert have shown the ability of this automated culture system to reliably detect as little as 1-10 CFU/mL [1, 2, 3 ,4]. The detection of the majority of bacterial species implicated in contamination of PCs can be realized within 24-48 hours. However the detection of slow growing bacteria like *Propionibacterium acnes* requires considerably more incubation time to be detected [5, 6, 7]. To circumvent this inconvenience a Real -Time (RT) PCR-based method was developed and validated to detect bacterial DNA in PCs; this method was published in previous studies [8, 9]. With this universal Real-Time quantitative PCR it is possible to detect analytically 1 CFU equivalent of bacterial DNA (i.e. 50 CFUequiv./ml) within 4 hours. The assay can be performed immediately after preparation of the PCs, before their release to hospitals or shortly before they are transfused. The current study was conducted to determine the optimal sampling time after PCs preparation using this PCR assay. To this purpose PCs were spiked with different concentrations of five microorganisms frequently associated with bacterial contamination of PCs. The presence of these bacteria was monitored over time with the PCR assay and by culturing in the BacT/Alert system (as reference method).

MATERIALS AND METHODS

Bacterial strains and culture conditions

The study was conducted with 5 microorganisms frequently associated with contamination of PCs. These included *Escherichia coli* (ATCC25922), *Bacillus cereus* (ATCC 11778), *Staphylococcus epidermidis* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853) and *Propionibacterium acnes* (clinical isolate). The bacterial strains were grown in Brain Heart Infusion Broth aerobically or anaerobically (in case of *P. acnes*). The bacteria were cultured until a turbidity of 0.5 McFarland (1.5×10^8 CFU/mL) was reached. From these suspensions serial tenfold dilutions were made in PBS. The suspensions were plated in triplicate on blood agar plates to determine the actual number of CFU/mL. From appropriate dilutions aliquots were drawn and used for inoculation of the PCs. The recovered concentrations were counted by plating and the results are presented in Table 1.

Inoculation of PCs:

For each bacterium six routinely prepared buffy coat-derived leukocyte reduced PCs (preparation is performed 22-24 hours after blood collection) units in plasma (of 300 mL each)[8] were pooled and then divided into 12 smaller pools of 150 mL. Three of these pools were used as negative controls (without spiking) and the other 9 were spiked with appropriate bacterial suspensions to obtain a final concentration of 1, 10 and 100 CFU per mL (3 separate 150 mL PCs pools per concentration). To monitor the presence of bacteria in these units, 10 mL aliquots (according to the European manufacturer's product insert) from each spiked PCs bag were transferred immediately after spiking (day 0), or after storage of 1, 2, 3, 6, or 7 days to both BacT/Alert standard aerobic and standard anaerobic culture bottles (bioMérieux, Boxtel, The Netherlands). Inoculation of the bottles on day 1, 2, 3, 6 or 7 was performed only when bacteria in one of the triplicate PCs bags were not yet detected after 1, 2, 3, 6 or 7 days of incubation (see flow diagram in Fig. 1). The inoculated culture bottles were incubated at 35^{o}C in the BacT/Alert either until the system gave a positive signal or for up to 7 days if they remained negative. As negative control aliquots of unspiked PCs were also used to inoculate the culture bottles of the BacT/Alert.

The bags with the remainder of the (spiked) PCs were stored at room temperature with continuous agitation for up to 7 days.

The presence of bacteria in the (inoculated) PCs bags was monitored by universal quantitative Real-Time PCR. For this application aliquots of 1 mL were drawn from all units during storage. Each PCs bag was sampled on day 0 (day of preparation of PCs and inoculation, this was 24 h after blood collection), day 1, day 2, day 3, day 6 and day 7 after inoculation and tested in parallel with the BacT/Alert. Inoculation of the BacT/Alert bottles was ended when a positive signal was noted from culture bottles of earlier time point inoculations.

Quantitative Real-Time PCR

DNA extraction

DNA extraction of samples drawn throughout storage at different time intervals from inoculated PCs was done with the MagNA Pure LC System (Roche diagnostics, Almere, The Netherlands). DNA was extracted from 200 µl aliquots using the MagNA Pure Total Nucleic Acid Isolation Kit according to the manufacturer's instructions. According to the standardized protocol, DNA extraction takes 90 minutes for 32 samples and the nucleic acids were eluted in a final volume of 50 µL. Extraction reagents were subjected to the same pretreatment as reported earlier [8].

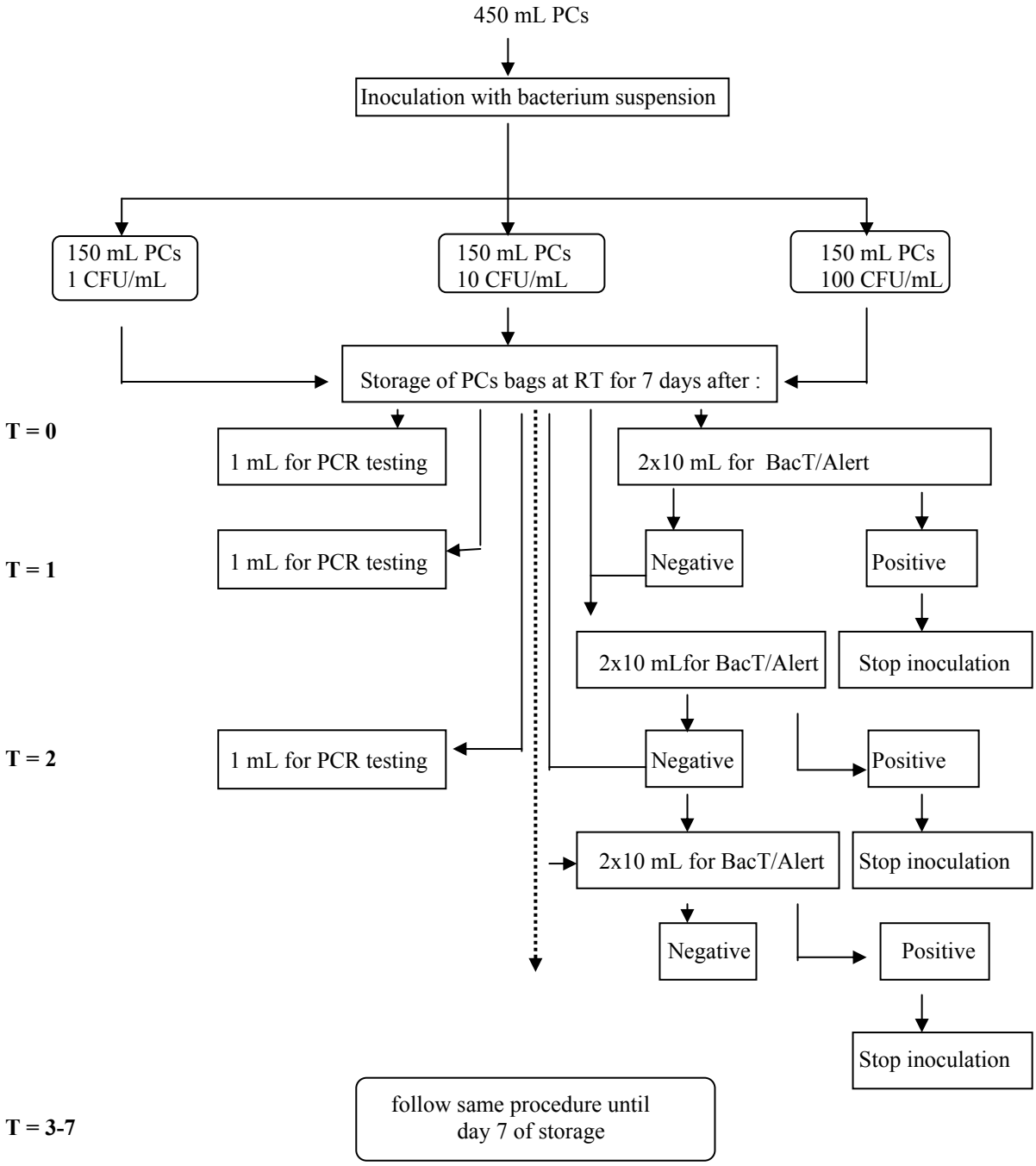

Figure 1: Flow diagram representing how a sample of PCs was inoculated with each bacterium species and time points of inoculations and sampling for BacT/Alert and PCR. For all three concentrations (1, 10 and 100 CFU/mL) the inoculations were conducted in triplicate. (T= Time in days)

PCR amplification:

The DNA was amplified following the same procedure as described earlier [8]. Briefly, the PCR was performed with universal primers and probe set. The sequence of the forward and the reverse primer were 5'- TCCTACGGGAGGCAGCAGT-3' and 5'- GGACTACCAGGGTATCTAATCCTGTT-3' respectively [9]. The sequence of the probe was (6-FAM)-5'- CGTATTACCGCGGCTGCTGGCAC-3' (TAMRA)[9].

Amplification was carried out in a total volume of 25 µL containing 12.5 µl 2X TaqMan Universal PCR Master Mix (consisting of AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference 1 and optimized buffer components, Applied Biosystems, Foster City, CA USA), 900 nM of each of the forward and the reverse primers, and 200 nM of the probe and 5 µL of template DNA.

As described before [9] the PCR mixture was subjected to digestion with the enzyme *Sau*3AI prior to the addition of template DNA, to eliminate possible contamination of PCR reagents with traces of bacterial DNA.

In addition to DNA extraction and PCR inhibition controls, no template controls (NTC) with sterile water instead of template DNA were included in each run. The thermal cycling program was as follows: 50^{o}C for 2 min, 95^{o}C for 10 min and 40 cycles of 95^{o}C for 15 min and 60^{o}C for 1 min. Real-time detection of the PCR products was performed in 90 minutes on the Sequence Detection System (ABI 7000, Applied Biosystems).

RESULTS

Inoculation of PCs

The recovered concentrations of bacteria are shown in Table 1. Based on the bacterial concentration of 0.5 McFarland the triplicate culturing results show that the initial inoculation amount were very reproducible. For *B. cereus* , *E.coli* and *S. epidermidis* the amount varied slightly under the amount of 10 and 100 CFU/mL. For *P.*

acnes and *P. aeruginosa* the amount of 10 and 100 CFU/ml were somewhat lower. For all bacteria the 1 CFU/mL inocula were correct whereas the amount of 10 CFU/mL showed the most differences.

Automated culturing

All day 0 inoculated samples cultured in the aerobic bottles were signalled positive by the BacT/Alert system, except when PCs were spiked with *P. acnes* (at all concentrations) and

with 1 CFU/mL of *E. coli*. The samples cultured in the anaerobic bottles gave a positive signal for all bacterial species tested, except when inoculated with 1 CFU/mL of *E. coli*, *P. acnes* or *P. aeruginosa*. Mean times to detection of each bacterial species in PCs by the BacT/Alert inoculated at day 0 are summarized in Table 2.

The bacteria were detected in both bottles within a mean time of 20.1 hours post-inoculation. Time to detection for most samples in the anaerobic bottles was faster or equal to the time to detection in the aerobic bottles (except for *P. acnes*). Overall, the data in Table 2 indicate that time to detection in both culture bottles of the BacT/Alert declined when the amount of the inoculated bacterium increased. Excluding results from *P. acnes*, a minimum and a maximum difference between ten-fold dilutions of other strains ranged between 0.8 and 2.52 hours respectively.

In case of *E. coli* at 1 CFU/mL no positive signal was detected in both culture bottles. However, when the culture bottles were inoculated with PCs samples of day 1, the signal became positive within 12.18 h in the aerobic bottle and 11.38 h in the anaerobic bottle (data not shown).

When Inoculation with *P. aeruginosa* was carried out on day 2 (on day 1 no inoculation was performed) a mean time to detection of 12.18 h in the aerobic bottle and 11.94 h in the anaerobic bottle was achieved (data not shown).

PCs spiked at 1 CFU/mL with *P. acnes* could only be detected in the culture bottles inoculated on day 6 of storage. All strains of bacteria spiked at an inoculum of 10 and 100 CFU/mL gave a positive signal after 1 day of storage of the PCs (i.e. 48 h after blood collection and 24 h after spiking).

During culturing for up to 7 days no positive signal was recorded by the BacT/Alert system for samples taken from all unspiked PCs (negative controls).

Quantitation by Real-Time PCR

The bacterial load determined over time of storage by quantitative RT- PCR for PCs spiked on day 0 at an initial inoculum of 1, 10 and 100 CFU/mL is presented in Table 3.

On day 0 all samples inoculated with 100 CFU/mL, that is 2 CFU equivalents/PCR, were detected by RT- PCR. In a previous study [10] the detection limit of the PCR assay was determined as 50 CFU/mL (i.e. 1 CFU equivalent/PCR), therefore the present results were in agreement with what was expected. This implies that numbers of bacteria below 50 CFU/mL (the detection limit of the PCR) cannot be detected. However with *B. cereus* and *E. coli* an

Table 1: Recovered concentrations of bacteria used to inoculate PCs.

		Recovered concentrations of 1, 10 and 100 CFU/mL		
Bacterium	source	1	10	100
B. cereus	ATCC11778	1 (1/3)	8-10 (3/3)	95-100 (3/3)
E. coli	ATCC25922	1-2 (2/3)	4-10 (3/3)	71-100 (3/3)
S. epidermidis	Clinical isolate	1-1 (2/3)	4-10 (3/3)	84-100 (3/3)
P. acnes	Clinical isolate	3 (1/3)	6-7 (3/3)	90-95 (3/3)
P. aeruginosa	ATCC27853	1 (1/3)	5-8 (3/3)	79-98 (3/3)

PCs were inoculated in triplicate with bacterial suspensions to achieve initial concentrations of 1, 10 and 100 CFU/mL. The upper and lower range of triplicate samples of the recovered concentrations is given. The number of reactive plates is given in parentheses.

inoculum of 10 CFU/mL was also detected. No amplification signal was observed for samples taken from control (unspiked) PCs (data not shown).

Detection of *E. coli* in the RT-PCR with an inoculum of 1 CFU/mL was positive for only 1 of the triplicate samples whereas none of the cultured *E. coli* gave a positive result for to up to 7 days with the same number of bacteria (and on day 0).

In general, from day 1 of storage (i.e. 48 h after blood collection) the presence of bacteria in PCs was detected by PCR in all tested samples and the corresponding bacterial load could be estimated. The quantitative results of the RT-PCR fluctuated and were not always in agreement with the increased inocula. The bacterial load of the culture however increased during storage of the PCs bags to reach a maximum in most cases on day 6 or 7 of storage according to the growth characteristics of the microorganism tested.

Finally, the RT-PCR procedure was completed within 4 hours including DNA isolation, amplification, detection and interpretation.

Table 2: Mean time to detection (hrs) in the BacT/Alert culture bottles of bacteria inoculated into platelet concentrates on day 0 at an inoculum of 1, 10, and 100 CFU/mL.

	Aerobic bottles		Anaerobic bottles	
	mean[a] (range)	reactive bottles[b]	mean (range)	reactive bottles
1 CFU/mL:				
B. cereus	15.7 (15.3 - 16.1)	2/3	14.3 (13.6 - 15.1)	3/3
E. coli	-[c]	0/3	-	0/3
S. epidermidis	20.1 (20.0 - 20.2)	2/3	19.2 (16.3 - 24.2)	3/3
P. acnes	-	0/3	-	0/3
P. aeruginosa	13.7 (13.1- 14.0)	3/3	-	0/3
10 CFU/mL:				
B. cereus	13.6 (13.2 - 13.4)	3/3	13.6 (12.3 - 14.4)	3/3
E. coli	12.3 (12.2 - 12.5)	3/3	11.5 (11.2 - 12.1)	3/3
S. epidermidis	19.3	1/3	18.3 (18.2 - 18.4)	2/3
P. acnes	-	0/3	102.3	1/3
P. aeruginosa	14.9 (14.3 - 15.5)	3/3	15.4 (14.5 - 16.2)	2/3
100 CFU/mL:				
B. cereus	12.1 (12.1 - 12.1)	3/3	11.9 (11.4 - 12.2)	3/3
E. coli	10.7 (10.6 - 11.1)	3/3	10.4 (10.3 - 10.6)	3/3
S. epidermidis	17.8 (17.4 - 18.1)	3/3	17.5 (15.2 - 20.2)	3/3
P. acnes	-	0/3	49.3 (48.2 - 50.4)	2/3
P. aeruginosa	13.1 (13.0 - 13.2)	3/3	12.8 (12.4 - 13.0)	3/3

[a] The values present the means of 3 units spiked with the same inoculum. The range (lower and upper limits) of detection is also given.
[b] Number of BacT/Alert bottles signalled positive.
[c] No growth of bacteria was detected.

DISCUSSION

Previously a quantitative Real-Time PCR method was developed to screen PCs [10] for bacterial contamination. The method was shown to be rapid, specific and highly sensitive [8]. To evaluate the application of this method and to determine the optimal time for sampling PCs, the current study was conducted in conjunction with the BacT/Alert as a reference.

On day 0 the presence of bacteria was detected at all inocula with the automated culturing system within 20.1 hours for all species tested, except for *E. coli* and *P. acnes* which were not found in initial inocula of 1 CFU/mL in both bottles. Although *P. acnes* is considered of no or low clinical significance, three clinical incidents of contamination of PCs with P. acnes have been reported by Schneider et al. [11]. Transfusion of these PCs caused septicaemia in the recipients. Therefore it seems important to detect this bacterial species to improve the safety of transfusion of PCs.

Table 3: Bacterial load (CFU equivalents/PCR reaction) determined at different time intervals by quantitative real-time PCR for PCs inoculated on day 0 at 1, 10 and 100 CFU/mL[a]

	Days post-inoculation of PCs					
Microorganism	t = 0	t = 1	t = 2	t = 3	t = 6	t = 7
1 CFU/mL :						
B. cereus	-[b]	$7.8x10^2$	$7.5x10^4$	$4.5x10^4$	$2.3x10^3$	$1.4x10^4$
E. coli	-	4.2	$1.2x10^2$	71.5	20.4	37.6
S. epidermidis	-	3.9	$1.2x10^4$	$1.4x10^2$	$6.2x10^6$	$3.4x10^6$
P. acnes	-	1.4	4.3	2.7	$4.7x10^3$	$4.2x10^3$
P. aeruginosa	-	ND[c]	$1.9x10^4$	$1.8x10^5$	$1.4x10^7$	$3.1x10^7$
10 CFU/mL :						
B. cereus	5.3	80.6	$3.4x10^5$	$7.2x10^4$	$2.1x10^3$	$2.3x10^4$
E. coli	3.1	42.4	$3.6x10^5$	$4.4x10^5$	$5.8x10^5$	$1.4x10^5$
S. epidermidis	-	5.7	$1.9x10^3$	$2.2x10^3$	$2.7x10^6$	$1.2x10^6$
P. acnes	-	2.7	3	$9.8x10^2$	$9.6x10^3$	$1.7x10^4$
P. aeruginosa	-	ND	29.1	$2.8x10^5$	$9.7x10^6$	$1.7x10^6$
100 CFU/mL :						
B. cereus	9.1	$4.8x10^3$	$3.6x10^5$	$1.9x10^4$	$1.3x10^3$	$2x10^4$
E. coli	5.8	62	$3x10^5$	$2.5x10^5$	$8.2x10^5$	$3x10^5$
S. epidermidis	6	$2x10^3$	$1.6x10^3$	$2.5x10^4$	$3x10^6$	$7.7x10^6$
P. acnes	15.1	4.7	3	$4.6x10^2$	$5.8x10^3$	$1.6x10^4$
P. aeruginosa	52.4	ND	$4.9x10^5$	$1.5x10^5$	$7.9x10^6$	$1.7x10^6$

[a] PCs were spiked in triplicate with each inoculum. The results are the means of triplicate inoculations. Bacteria were detected in all triplicate samples, except *E. coli* at an inoculum of 1 CFU/mL that was detected in only one sample at day1, 2, 3 and 6.
[b] No amplification signal was detectable. [c] ND: not determined.

On day 1 of storage of the PCs, the automated culturing system recorded the presence of all strains of bacteria at all concentrations, except *P. acnes* at 1 CFU/mL, which was detected only on day 6 of storage in the anaerobic culture bottle.

The use of a quantitative Real-Time PCR to monitor the presence and growth of bacteria in PCs demonstrated the ability of this assay to detect reproducibly all bacteria on day 0 (day of PCs preparation and spiking) when an inoculum of 100 CFU/mL in the original PC bag, was applied (Table 3). The detection of less than 100 CFU/mL was only possible for *B. cereus* and *E. coli*. These species have a short generation time (20-30 min in broth) and could therefore reach the number which can be detected by PCR more easily. However other species like *S. epidermidis* and *P. aeruginosa* can also double in 30 minutes. Knowing that the time between inoculation and sampling of PCs did not exceed 2 hours, it is probable that these bacteria have an extended proliferation time in PCs A possible bacteriocidal effect from the PCs on these strains might be responsible for this effect resulting in a reduction of the amount of viable bacteria. The remaining viable organisms will grow subsequently in the following days. On

the other hand, the detection depends not only on the detection limit of the PCR assay but also on the probability of the presence of a bacterium in the sample taken from the PCs bag. Although the analytical sensitivity of the RT-PCR is 1 CFUequivalent /PCR (50 CFU equivalents/mL) the clinical sensitivity in the PCs may be somewhat different due to the effect of the specimen. Based on the maximum sensitivity only the 100 CFU/mL inoculum would be detectable. However because of a probabilistic effect also some 10 CFU/mL samples were positive. This also explains the fact that there is no difference in quantification between the 10 CFU/mL and the 100 CFU/mL quantitative results since this is both at the limit of the sensitivity.

Remarkably *P. acnes* could be detected directly with the PCR assay after inoculation of 100 CFU/mL on day 0, whereas with automated culturing a positive signal was recorded after 49.3 hours of incubation in the anaerobic bottles only. Although growth conditions (medium, temperature) in the culture bottles of BacT/Alert are more favourable for growth of most bacteria than when they are stored in PCs at room temperature, the detection with PCR was faster than in the BacT/Alert. This demonstrates the sensitivity of the PCR assay in detecting slow growing microorganisms.

With the PCR assay all tested bacteria could be consistently detected one day after spiking, regardless of the size of the inoculum. The bacterial load increased as the storage time was prolonged to achieve a maximum when bacteria enter the stationary phase of growth. Compared to other bacteria, *P. acnes* exhibited slower growth and the bacterial load reached no more than approximately $2x10^4$ CFU equivalents/PCR at the end of 7 days of storage. This is in accordance with the results of the BacT/Alert where this microorganism showed the longest time to detection.

Interestingly, all PCs contaminated with 1 CFU/mL could be reliably detected by PCR after one day of storage (day 1) in all tested samples except *E. coli* which was detected only in one of the three inoculated samples. In BacT/Alert this amount required an incubation time of at least 20.1 hrs to reach a detectable threshold. The efficacy of detection with PCR may offer the possibility of removing contaminated PCs prior to issue to hospitals. In this case two advantages of this approach can be mentioned being the time to results and standardized procedures. As described in the methods section the whole procedure (DNA isolation, amplification and detection) is standardized and is completed within 4 hours (extraction 90 min for 32 samples, amplification 90 min and analysis 5 min, sampling and pipetting 30 min).

The whole procedure is carried out in closed systems with ready-to-use DNA isolation reagents and commercial RT-PCR master mixes. In addition, the use of Real Time PCR prevents any post-amplification handling. Therefore, in general, the RT-PCR is suited for routine performance. However it should be mentioned that PCR detects DNA from both viable and non-viable bacteria. The detection of non-viable bacteria by PCR may affect the specificity of the assay. Nevertheless in routine this is of no importance as long as the sensitivity of PCR is good enough to use this screening test as a qualitative criterion to release PCs.

As the input of a sample in the RT-PCR was approximately 100 fold less than in the automated culturing system, RT-PCR has the potential to become more sensitive when the input volume will be increased.

In summary, the findings of this study show that the PCR assay has the advantage of providing results in a short time (within 4 hours):. Contamination can be detected after preparation of PCs, though the initial amount of some bacteria is low (less than 1CFU equivalent/PCR reaction). Based on the available literature the initial contaminating amount of bacteria is thought to be in the order of 1-10 CFU/mL blood [14]. In some cases (as detailed above) this amount could be missed if testing is performed immediately after preparation of PCs since the detection level of the RT-PCR is 50 CFU equivalents/ml. Therefore to assure optimal detection of all relevant bacterial species by PCR, it is reasonable to sample (routinely prepared) PCs at least one day after preparation (i.e. 48 hours after blood collection). At this time point the PCR has the potential to detect bacteria present in PCs even at low initial concentrations.

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Chapter 7

Amplified fragment length polymorphism analysis of *Propionibacterium* isolates implicated in contamination of blood products

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ABSTRACT

Propionibacterium acnes is implicated in most cases of bacterial contamination of platelet concentrates (PCs). To determine the source of contamination, amplified fragment length polymorphism (AFLP) analysis was applied. This DNA fingerprinting technique was used to study the molecular relatedness of 44 isolates derived from 22 PCs and 22 corresponding red blood cells concentrates (RBCs) from the same whole blood donations. The AFLP results together with sequencing analysis of the 1200 bp of the 16S ribosomal RNA gene revealed the existence of three main groups: two groups (group 2 and 3) (55%) consisting of isolates that did not originate from skin flora and another group (group 1)(45%) comprising bacteria belonging to the skin flora. This latter group showed complete homology with reference strains of *P. acnes*. Therefore these isolates can be considered as *P. acnes* strains. In contrast, contaminants from group 2 and 3 were shown to be molecularly unrelated to *P. acnes* that belongs to the surface of the skin.

The AFLP is reproducible and gave invaluable information about the nature of *Propionibacteria* contaminating PCs. To gain more insights into the source of contamination, this technique could be exploited in further studies to determine the molecular relatedness of different bacteria commonly found in blood products.

INTRODUCTION

Despite major efforts to reduce the rate of transfusion-transmitted bacterial infections, contamination of blood products with bacteria still occurs. Platelet concentrates (PCs) are frequently associated with transmission of bacterial infections. Yet, little is known about the source and mechanisms of the contamination, although the donor has often been recognized as the source. Contamination can either originate from the skin surface during venipuncture, caused by inadequate disinfection of the donor arm at the phlebotomy site or from transient bacteraemia (Blajchman *et al*, 2004).

The majority of the microorganisms implicated in contamination are part of the skin flora. As shown in various studies, *Propionibacterium* is the most frequently implicated organism (Mohammadi *et al*, 2005). *Propionibacterium* is a Gram-positive, non-spore forming anaerobic bacterium. This opportunistic pathogen is found predominantly in the cutaneous flora. Since this bacterium is considered to be of low clinical significance (Wilson *et al*, 2004), its presence in blood is usually interpreted as (extraneous) contamination and not as the result of true infection. However, different studies have reported the involvement of *Propionibacteria* spp. in serious infections such as endocarditis, post-operative endophthalmic infections (Hykin *et al*, 1994; Jakab *et al*, 1996; LePage *et al*, 2003), osteomyelitis, arthritis and sarcoidosis (Ishige *et al*, 1999). Recently, a case study reported by Schneider *et al*. (2000) documented the clinical sequelae of three incidents of platelet contamination by *P. acnes*. The authors assumed that contamination could be the result of insufficient skin disinfection at the venipuncture site during the phlebotomy process.

In general, all these cases emphasise the importance of knowing whether the detection of *Propionibacterium* in PCs is mere contamination or represents a genuine infection.

In the present study, amplified-fragment length polymorphism (AFLP) analysis, was used to study possible sources of contamination of PCs with *Propionibacteria*. In brief, this technique combines simultaneous restriction-ligation and selective PCR amplification (Vos *et al*, 1995; Savelkoul *et al*, 1999). Genomic DNA is digested with two restriction enzymes, and then double-stranded oligonucleotide adapters are ligated to the DNA fragments. Subsequently selective PCR amplification is performed with adapter-specific primers. At the 3'ends of these primers selective nucleotides (one to three) are included to amplify a subset of the ligated fragments.

AFLP has been widely applied to identification and genotyping of various organisms. Because of its high discriminatory power and reproducibility this DNA fingerprinting method was employed to study the molecular relatedness of *Propionibacterium* species responsible for contamination of PCs and corresponding red blood cells concentrates (RBCs) from the same whole blood donations.

MATERIALS AND METHODS

Bacterial strains:

Clinical strains of *Propionibacterium* spp. isolated from contaminated platelet concentrates (PCs) and corresponding (i.e from the same whole blood donations) red blood cells concentrates (RBCs) were obtained from the department of Bacteriology of the Slotervaart Hospital (Amsterdam, The Netherlands). These strains were cultured from PCs and RBCs produced at the blood bank and sent to the hospital for confirmation and identification of the bacteria implicated in the contamination. Processing of PCs and RBCs and screening PCs for bacterial contamination were done according to the procedure detailed in a previous study (Mohammadi *et al,* 2005). The presence of bacteria in RBCs was assessed using the same procedure as described previously for PCs. In brief, standard aerobic and anaerobic culture bottles of the automated culture system (BacT/Alert) are inoculated with 5 to 10 mL aliquots of RBCs. The aliquots are incubated in the BacT/Alert until a positive signal was recorded or for up to 7 days if they stayed negative. Positive culture bottles were subcultured for confirmation and identification of microorganisms. Positive units of PCs and corresponding RBCs were recalled from inventory and/or from hospitals (if already issued).

For this study 44 strains cultured from PCs and corresponding RBCs (22 from PCs and 22 from RBCs) were obtained. In addition, 6 strains from PCs without RBCs counterparts and 5 strains from RBCs without PCs counterparts were included. The strains were collected during one year.

Other clinical laboratory strains of *Propionibacterium acnes* (n = 11) were obtained from the Department of Medical Microbiology and Infection Control of the VU University Medical Centre (Amsterdam, The Netherlands). These bacteria originating from normally sterile body fluids (such as cerebrospinal fluid and blood) of patients with severe infections were collected over 3 years. The isolates were cultured on blood agar at 37^{o}C under anaerobic conditions. These isolates were identified by conventional biochemical techniques. Seven other strains

representing different transient bacterial species of *Propionibacteria* from skin flora of healthy volunteers were also included.

Propionibacterium acnes (DSM 1897, ATCC 6919), *Propionibacterium avidum* (DSM 4901, ATCC 25577), *Propionibacterium granulosum* (DSM 20700, ATCC 25564) and *Propionibacterium propionicus* (DSM 43307, ATCC 14157) were included in this study as reference strains. These strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) and cultured under anaerobic conditions as specified by the manufacturer.

DNA extraction:

Prior to extraction of DNA , pure bacterial colonies were resuspended in 100 µL TE (10mM Tris-HCL, 1mM EDTA, pH 8.0) buffer and adjusted to match a turbidity of 0.5 McFarland. The suspension was then incubated with 10 mg/mL lysozyme at 37^{o}C for at least 1 hour. Thereafter DNA was isolated following the tissue protocol of the QIAmp DNA mini kit (QIAGEN, GmbH, Hilden, Germany). Finally, the nucleic acid was eluted in 100 µl AE buffer of the extraction kit and stored at -20^{o}C until needed.

Genotyping of *Propionibacterium* strains:

Amplified fragment polymorphism analysis

Amplified fragment polymorphism (AFLP) analysis was applied for genotyping of *Propionibacterium* strains. DNA was restricted with two enzymes, and two adapters were ligated to the restriction sites in the same reaction. The reaction mixtures consisted of 10 ng DNA, of 1xT4 DNA ligase buffer, 0.5 M NaCl, 0.5 µg bovine serum albumin, 2 pmol of the *Eco*RI adapter (Isogen Bioscience BV, Maarssen, The Netherlands), 20 pmol of the *Mse*I adapter (Isogen Bioscience), 80 U of T4 DNA ligase, 0.2 U of *Eco*RI, 1 U of *Mse*I. After incubation at 37^{o}C during 3 hours, the mixtures were diluted 1:20 in 0.1 X TE buffer. All other enzymes were purchased from New England Biolabs (Beverly, Mass.)

For amplification of the restriction fragments, five µL of the diluted mixture was added to 5 µL of PCR mixture which consisted of 1 x PCR buffer (Applied Biosystems, Foster city, CA), 2 mM dNTPs (Promega Benelux, Leiden, The Netherlands), 15 mM $MgCl_2$ (Applied Biosystems) , 1 U Ampli*Taq* DNA polymerase (Applied Biosystems), and 20 ng of *Eco*-A

primer (5'- GACTGCGTACCAATTCAC-3') and 60 ng of *Mse*-C primer (5'-GATGAGTCCTGAGTAAC-3'). *Eco*-A was fluorescently labelled with FAM (Eurogentec, Maastricht, The Netherlands). Amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 2 min at 72^{o}C, followed by 12 cycles of 30 sec at 94^{o}C, 30 sec at 65^{o}C and 1 min at 72^{o}C and then 23 cycles of 30 sec at 94^{o}C, 30 sec at 56^{o}C and 1 min at 72^{o}C, ended by a single extension at 72^{o}C for 1 min. Before analysis on an ABI Prism 3100, 2.5 µL of each PCR product was added to 22 µL Hi-Di formamide and 0.5 µL GeneScan-500 ROX standard (Applied Biosystems). Data were analysed with the BioNumerics software package, version 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated with Pearson correlation and dendrograms were obtained by the unweighted -pair group method using arithmetic averages (UPMGA) clustering.

Amplification and sequencing

PCR was performed using the universal primers described in a previous study which target a conserved region of 16S ribosomal DNA (Mohammadi *et al*, 2005). A 20 µL PCR mixture consisting of 2.5 µL 10xPCR buffer, 0.5 µL 10mM dNTPs, 1.5 µL 25 mM MgCl2, 25 pmol of each of the forward (5'-TCCTACGGGAGGCAGCAGT-3') and reverse (5'-GGACTACCAGGGTATCTAATCCTGTT-3') primer (Eurogentec**)**, 0.2 µL Amplitaq gold (5 U/µL) and 5 µL template DNA was amplified under these conditions: 10 min at 95^{o}C followed by 30 cycles of 30 sec at 95^{o}C, 30 sec at 60^{o}C and 45 sec at 72^{o}C and a final step of 10 min at 72^{o}C. PCR products were analysed on 2% agarose gel in 1xTBE buffer (Life technologies ltd, Paisely, Schotland**)**. After amplification the PCR products were purified using the Quiaquick PCR purification kit (Qiagen).

The purified products were sequenced using primers covering a 466 bp fragment from the 16S rDNA. The primers gd1 (5'-TGCTTTCGATACGGGTTGAC-3') and bak 4 (5'-AGGAGGTGATCCARCCGCA-3')(Dasen *et al*, 1998) were also employed. These primers amplify a *Propionibacterium*-genus 900 bp fragment. Primers were obtained from Eurogentec (Maastricht, The Netherlands).

Sequencing was performed using Big Dye terminator sequencing kit (Applied Biosystems). The program consisted of: 10 sec at 96^{o}C, 5 sec at 56^{o}C and 4 min at 60^{o}C for 25 cycles. The sequence products were purified and then subjected to analysis on an ABI 3100 automated DNA sequence analyser (Applied Biosystems). Sequence homology analysis was done by using the BLAST program**.**

RESULTS

Reproducibility of the AFLP profiles

The reproducibility of the AFLP results was assessed by subjecting duplicate samples to the same extraction, restriction/ligation and amplification procedures. AFLP patterns of all duplicate samples clustered with a similarity level of more than 90% (Fig. 1). Based on these results a cut-off value of 90% was chosen for definition of identical strains.

Definition of windows of similarity

Analysis of unrelated clinical laboratory isolates of the species *P. acnes* showed that the patterns of these strains were homologous among each other: patterns clustered in a window of similarity between 55 to 90%. The ATCC strains *P. avidum*, *P. granulosum* and *P. propionicus* separated at a similarity level of less than 30%.

Based on these results 4 windows of similarity were determined: the first between 90 and 100% defines identical strains within a species, the second between 55 and 90% defines different strains of the same species, the third between 30 and 55% defines related species (possibly different subspecies) and the fourth below 30% distinguishes different species.

AFLP analysis of the isolates

To determine the precise length of the PCR fragments the ABI Genescan analysis software was used. AFLP patterns were analysed for the fragments with sizes between 200 and 500 base pairs. The AFLP profiles were then imported in the Bionumerics software package. After normalization (to the internal size standard that was included in each sample)(see Materials and Methods), the degree of similarity between AFLP profiles were determined by using the Pearson correlation coefficient. Clustering was performed with the UPGMA method.

The primer combinations *Eco*-A and *Mse*-C generated the dendrogram shown in Fig. 2. Based on this dendrogram, the isolates described in Materials and Methods could be classified into three main groups. Group 1 comprised all *P. acnes* strains isolated from patients with bacteraemia (n = 11), laboratory isolates from healthy volunteers (n = 7) and isolates from PCs (n = 10) and RBCs (n = 14). Group 2 comprised isolates from 10 PCs and 7 RBCs (derived from the same donations), while group 3 enclosed the remainder of the tested isolates (7 PCs and 6 RBCs). One PC isolate (P33) exhibited a totally different banding pattern, which indicated that this strain belonged to a different species. Interestingly, the strains isolated from

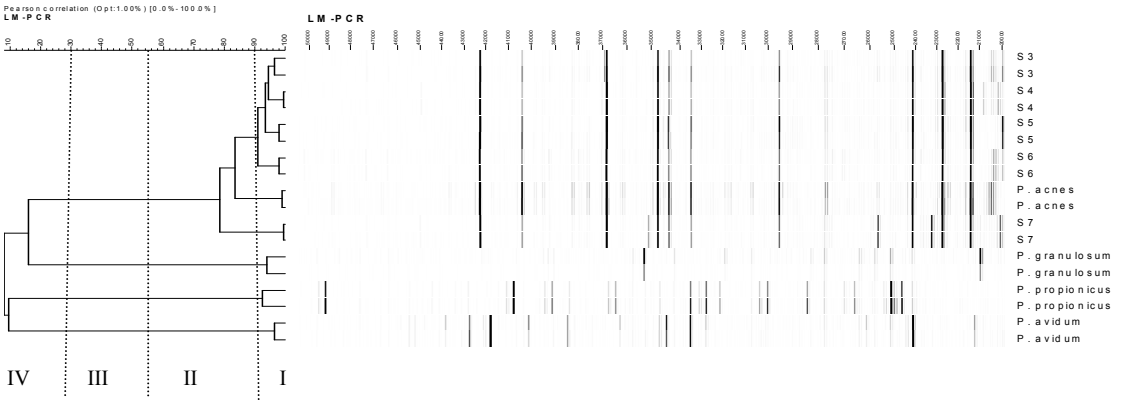

Figure 1. Assessment of the reproducibility of the AFLP protocol. Duplicate samples derived from the skin flora (denoted with S) were subjected to the same procedures. The banding patterns were obtained by using the ABI Genescan analysis software. After normalization AFLP profiles were compared using the Pearson correlation coefficient and clustered by the unweighted pair group with mathematical average (UPGMA) method. All duplicates clustered with similarity levels higher than 90%. I: 90 to 100% homology illustrates identical strains, II: 55 to 90% homology indicates different stains within the same (sub)species, III: 30 to 55% homology demonstrates possible different subspecies, IV: less than 30% homology indicates different species within the same genus.

patients with bloodstream infections and skin contaminants formed a cluster (group 1) that was clearly separate from the majority of the isolates derived from the blood products, which clustered in groups 2 and 3.

As expected the cutaneous species (ATCC reference strains) *P. acnes*, *P. avidum*, *P. granulosum*, and *P. propionicus* differed from each other and exhibited different banding patterns (window IV). All isolates in group 1 clustered with the reference strain *P. acnes* with a similarity level between 55 and 90%. Therefore these isolates can be considered as *P. acnes* strains.

Sequence analysis of 1200 bp of the 16S ribosomal RNA gene of representatives of this group showed 99% homology with *P. acnes* (GenBank accession number AB097215) and 99% homology with *P. acnes* ATCC 6919 (GenBank accession number AB042288). Two bases did not match those of these references (Table 1, P19). This suggests that these were possibly distinct strains within the same species.

Based on AFLP profiles the isolates categorized as group 2 and 3 showed 55 to 90% homology, which implies that these bacteria belong to the same (sub)species. Sequence analysis of these strains revealed 97 to 98% homology with the 16S ribosomal RNA gene of

P. acnes (GenBank accession number AB097215) and *P. acnes* ATCC 6919 with 13 and 11 mismatches respectively.

Among the isolates that were classified in group 2, 7 that were derived from PCs were identical (90% homology) to their RBCs counterparts. Isolates P2, P5 and P7, which had no RBCs counterparts separated also with similarity levels ranging between 55 and 90%.

Within group 3, 5 of the 7 PCs isolates showed banding patterns identical (90% homology) to those derived from RBCs originating from the same donations. Only isolates P12 and R18 clustered separately from their counterparts R12 and P18 which clustered in group 1.

In summary, 13 PCs isolates were identical to their counterparts in RBCs. On the other hand, 9 PCs isolates differed at the strain level from the isolates found in corresponding RBCs.

Sequence analysis of the 16S ribosomal DNA suggested that the isolates in group 1 were most closely related to the ATCC strain 6919. Compared to the nucleotide sequence of strain ATCC 6919, 2 mismatches were observed for a representative of group 1 (P19), 1 to 11 mismatches for representatives of group 2 and 2 to 7 mismatches for representatives of group 3 (see Table 1). These nucleotide variations could indicate the existence of a subspecies of *P. acnes* at the same similarity level as *P. avidum* and *P. granulosum.*

Sequence homology analysis of P33 revealed a 97% homology with both *P. acnes* (18 mismatches) from the GenBank and *P. acnes* ATCC 6919 (17 mismatches). This implies that this isolate is a different species. For this PC isolate, no related RBC isolate was available for AFLP typing and sequencing analysis.

Overall, the DNA fingerprints of the isolates together with sequencing analyses demonstrate that only 45% of all cases of contamination of PCs in the studied collection are caused by *P. acnes* strains most closely related to the strains derived from skin surface flora. This suggest that the strains (or subspecies) of *P. acnes* that account for most instances of contamination of PCs and corresponding RBCs are derived from other sources.

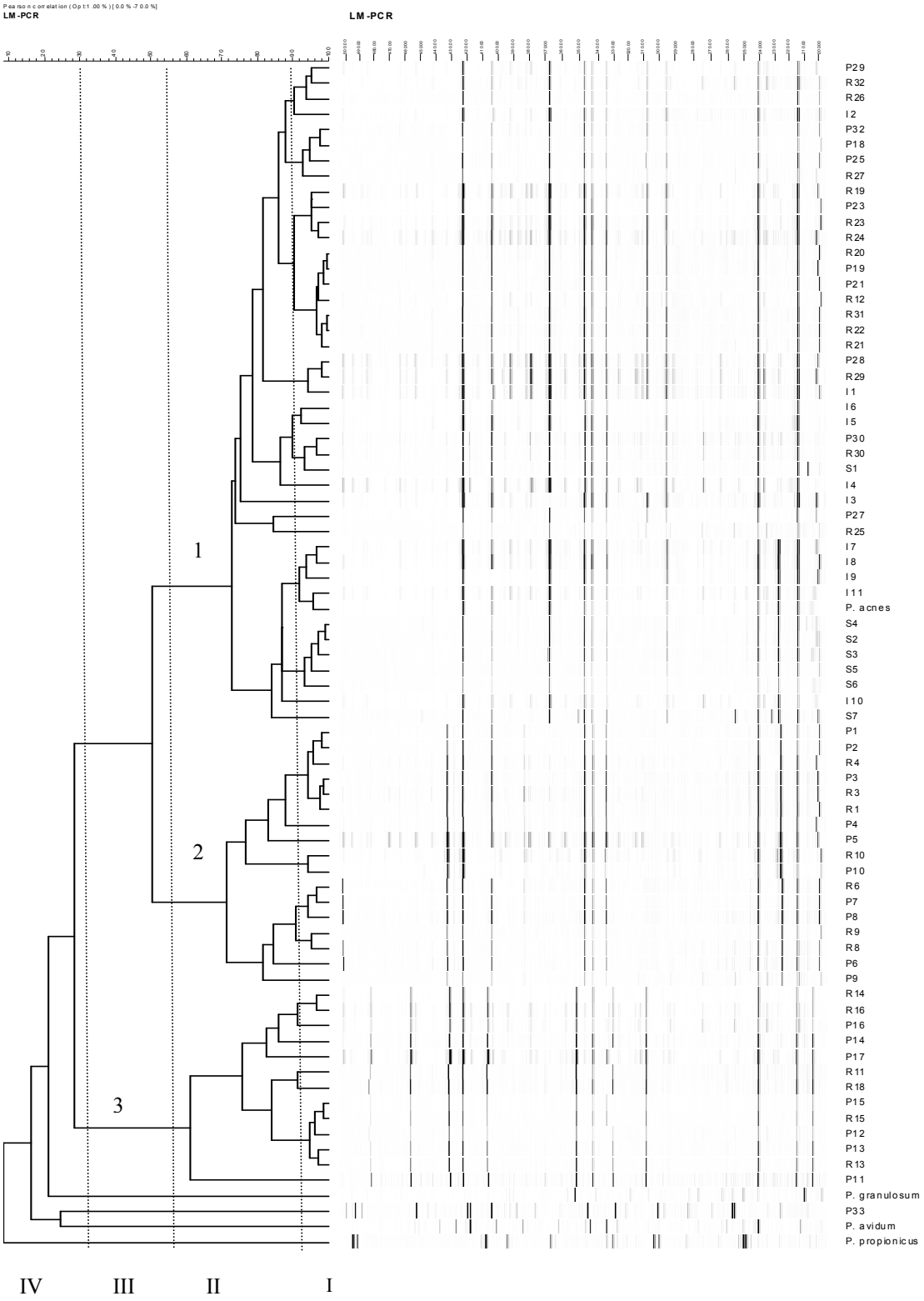

Fig.2. Unweighted - pair group method using arithmetic averages (UPGM) dendrogram derived from the AFLP patterns of all 55 isolates of platelet concentrates (PCs) and red blood cells concentrates (RBCs). The analysis was performed between 200 and 500 bp. PCs and related RBCs are denoted with the same number beginning with P and R respectively. P2, P5, P7, P17, P28 and P33 have no RBCs counterparts. R20, R22, R24, R26 and R31 have no PCs counterparts. The isolates from the bloodstream infections are represented with I and the isolates from skin flora with S. The reference strains are also given. I: 90 to 100% homology illustrates identical strains, II: 55 to 90% homology indicates different stains within the same (sub)species, III: 30 to 55% homology demonstrates possible different subspecies, IV: less than 30% homology indicates different species.

Table 1. Comparison of the nucleotide sequences of the 16S RNA gene of ATCC 6919 and *Propionibacterium* spp. isolates from PCs.

		Isolates from platelet concentrates*					
Nucleotide Position	Reference strain ATCC 6919	P1	P3	P14	P16	P19	P33
433	g	.	.	.	.	.	a
434	a	.	.	.	.	.	g
435	c	.	.	.	.	.	t
445	c	.	.	.	.	.	a
449	t	.	.	.	.	.	a
459	t	.	.	.	.	.	c
471	c	.	g	.	.	.	.
472	g	.	c	.	.	.	.
475	g	.	c	.	.	.	.
548	c	.	g	.	.	.	.
549	g	.	c	.	.	.	.
574	c	.	.	.	.	.	g
581	a	.	.	.	.	c	.
609	g	.	.	.	.	.	a
628	c	.	.	.	.	.	.
640	c	.	.	.	t	g	.
655	a	.	.	.	c	.	.
671	c	.	.	.	g	.	.
673	t	.	a	.	.	.	.
674	c	.	.	t	.	.	.
694	t	.	.	.	.	.	c
697	g	.	.	.	c	.	.
710	g	.	.	.	a	.	.
719	a	.	.	.	n	.	.
827	a	g	.	.	.	.	.
947	a	.	.	.	.	.	t
976	c	.	.	.	.	.	t
985	g	.	.	.	.	.	a
997	a	.	.	.	.	.	g
1011	a	.	.	.	.	.	g
1012	g	.	.	.	.	.	a
1134	g	.	.	.	.	.	a
1226	c	.	a	.	.	.	t
1243	c	.	.	t	t	.	.
1282	a	.	g	.	.	.	.
1297	g	.	t	.	.	.	.
1305	a	.	t	.	.	.	.
1442	g	.	t	.	.	.	.

* Representatives isolates of group 1, group 2 and group 3 were aligned with the reference strain *P. acnes* from the GenBank.Dots indicate the same nucleotide residues as the ATCC strain.

DISCUSSION

Propionibacterium spp. is frequently associated with contamination of PCs. In an attempt to identify the source of bacterial contamination of blood products with *Propionibacterium* spp. AFLP fingerprinting was applied. This typing method is based on the comparison of patterns of DNA restriction fragments derived from the total bacterial genome. With this fingerprinting method the molecular relatedness of *Propionibacteria* found in PCs and their corresponding RBCs was analysed. Based on the AFLP profiles, isolates of PCs and corresponding RBCs could be grouped into three main AFLP profile groups that were designated group1, group 2, and group 3 (Fig.2). In group 1 both fingerprinting and sequencing results revealed (almost) complete homology of PCs and RBCs isolates with bacteria isolated from skin (clinical laboratory strains of *P. acnes*). Therefore it is conceivable that these isolates originated from skin flora of the donor. The isolates of this group may have been introduced in the blood bags during the collection procedures, i.e. by inadequate disinfection of the donor's arm or the introduction of skin surface commensals through the needle during venipuncture.

Representatives of group 2 and 3 showed clearly different banding patterns and sequencing results indicated that the contaminants are distinct from *Propionibacteria* belonging to the transient skin flora. The isolates varied also among each other. This was also observed by Kunishima *et al*. (2001) when they analysed *P. acnes* isolated from PCs by using random amplified polymorphism DNA (RAPD).

Of note is the difference at strain level between the isolates derived from PCs and corresponding RBCs (from the same donations). This difference indicates that these contaminants do not originate from whole blood but are introduced in PCs and RBCs separately. It is possible that contamination of these blood products occurred during sampling of the PCs to inoculate both BacT/Alert culture bottles. This procedure is part of the QC of PCs and is carried out to screen PCs for bacterial contamination.

The difference could also be due to the fact that PCs and RBCs are stored in different media and temperatures (PCs at 22^{o}C and RBCs at 4^{o}C). It is conceivable that the storage conditions favour the growth of one bacterial strain more than another if both originate from the same source.

Provided that the contaminants of group 2 and 3 were shown to be molecularly unrelated to *P. acnes* derived from the surface of the skin, it is likely that they originate from another habitat.

The source of these contaminants is still unclear. Since isolates from PCs and related RBCs clustered in the same main group (except for isolates from 2 PCs), it is apparent that they originated from whole blood of the donor and not from extraneous contamination as generally assumed. In the later case, the differences between the various isolates would be larger.

The contaminants of PCs and RBCs could also be derived from deeper layers of the skin containing the sebaceous glands and hair follicles. During the phlebotomy process, these bacteria could be introduced in the collected blood through the needle.

Up to now, several strategies have been followed by many blood centres to reduce the rate of bacterial contamination of blood products. Among these are the improvement of donor-arm disinfection methods and the diversion of the first 10-20 mL of blood from the collection bags. Since the majority of contaminants are not skin (surface) derived, it is probable that these methods would not result in entire elimination of contamination of blood products with *Propionibacterium* spp. This means that when these strategies are applied only skin derived commensals (45%) may be removed, but the majority (55%) is already present in the collected whole blood or introduced inadvertently during manufacturing.

The findings of this study are in accordance with the results obtained by McDonald *et al*. (2004), who studied the effect of diversion of the initial 20 mL blood from the collection bag and of an improved donor-arm disinfection method in reducing bacterial contamination of donated whole blood. They reported a reduction of 47% when diversion of the initial aliquots of blood was applied. The reduction reached 57% when diversion was done in combination with an improved donor-arm disinfection procedure. A reduction of contamination (from 0.35% to 0.21%) was also reported by De Korte *et al*. (2002) after diversion of the initial 10 mL aliquots of blood. Remarkably, in their study only a reduction in the rate of contamination with *Staphylococcus* spp. was observed. The rate of contamination with *Propionibacterium* spp. remained similar. In view of this and of the AFLP results it is apparent that improving these methods will not result in eradication of contamination of blood products. Therefore, it might be necessary to focus on other steps in the collection and manufacturing of blood especially on the needle used in the first step of blood collection.

In addition, since PCs and RBCs from the same donation may contain different bacterial strains as alluded to above, it might be necessary to take sufficient precautions to prevent inadvertent contamination after collection.

In conclusion, the AFLP technique provided reproducible and invaluable epidemiological information for *Propionibacteria* found in blood products, although the source of

contamination of PCs with these bacteria was not completely traced. This information cannot be obtained when using for example only sequencing of the 16S ribosomal RNA gene. The difference resides in the fact that AFLP patterns represent the whole genome, whereas sequencing covers only the 16S ribosomal RNA gene.

The AFLP method appears as very promising for further studies to determine the source and the molecular relatedness of bacteria implicated in contamination of blood products. Such studies would help blood centres in designing measures to prevent or to reduce the rate of bacterial contamination of cellular blood products.

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Chapter 8

Quantitation of 16S ribosomal DNA and RNA as a new approach to monitor the presence and state of viability of *Escherichia coli*

ABSTRACT

A method, based on quantitation of DNA and RNA, was developed to detect not only the presence but also the viability of *Escherichia coli*. For reproducible quantitation a reliable and stable standard was needed. Therefore, an armored RNA standard with 16S rRNA of *E. coli* as target was constructed and assessed in real-time PCR. With this standard the analytical sensitivity of the real-time PCR was 14 copies/PCR. The standard was further employed to quantify 16S ribosomal DNA and RNA during the growth of *E. coli* in broth as well as in platelet concentrates (PCs). DNA was detected during the whole life cycle of bacteria: the amount of DNA increased in the logarithmic phase, remained stable during the stationary phase and declined slowly during the death phase.

The quantity of 16S rRNA in the cell culture correlated with the viability of bacteria: the amount of RNA increased during the logarithmic phase as the amount of DNA increased, it remained stable during the stationary phase and then diminished much more rapidly than DNA to undetectable levels, which coincided with loss of viability.

The effect of two antibiotics on the 16S rDNA and 16S rRNA content was also assessed. When bacteria were exposed to chloramphenicol, a bacteriostatic drug, during the logarithmic phase of growth, the amount of RNA stayed constant for two hours, declined thereafter and then disappeared completely. In contrast, the DNA content showed a slight decrease but remained detectable. Upon further incubation in antibiotic-free medium, the bacteria started to grow again and RNA became detectable again. A similar effect was also observed when bacteria were treated with amikacin, albeit that due to bactericidal effect of this antibiotic, RNA disappeared faster than with chloramphenicol and growth did not resume in antibiotic-free medium. This could be monitored by the total lack of reappearance of RNA. This approach may prove useful to monitor the effect of antibiotics during treatments of infections with bacterial pathogens.

INTRODUCTION

Ribosomal, messenger and transfer RNA can be used as indicators of viability of bacteria. To detect RNA sequences in clinical samples various techniques such as reverse transcription (rt)-PCR, nucleic acid sequence-based amplification (NASBA) and branched chain DNA have been adopted by most molecular diagnostics laboratories (1, 2, 3). In addition to the inclusion of internal controls to monitor the process of extraction, reverse transcription, amplification, and detection these RNA-based methods require the use of standards for quantitation. To date, most of the standards are developed only for assays to detect viral RNA and to assess the effect of antiviral therapies. However, such assays would also be very valuable to monitor the response to antibiotics when treating patients for infectious diseases with bacterial pathogens. Recently, armored RNA controls were commercially developed as internal controls for viral Real-Time PCR assays (4). Armored RNA permits the production of ribonuclease-resistant RNA particles (5). In the present study this technology was exploited to construct an RNA standard to quantify 16S ribosomal RNA of *E. coli*. Simultaneous quantitation of DNA and RNA was subsequently used as a new approach to monitor not only the presence but also the viability of bacteria both in a bacterial cell culture as well as in platelet concentrates (PCs) for transfusion.

MATERIALS AND METHODS

Construction of 16S Armored RNA standard

RNA from a suspension of *E. coli* (ATCC 25922) was isolated with the RNAqueous kit (Ambion Inc, Austin, Texas USA) following the instructions of the supplier. The RNA was converted to cDNA with RETROscript kit (Ambion Inc.). The rt- reaction was performed in a total volume of 40 µl with Random Decamers according to the recommendation of the manufacturer. The universal forward primer (5'-TCCTACGGGAGGCAGCAGT-3') and the reverse primer (5'-GGACTACCAGGGTATCTAATCCGTT-3') were then used in the PCR reaction to amplify a highly conserved 466bp fragment of the bacterial 16S rDNA (6). The resulting PCR product was cloned into pGEM-T easy vector (Promega) as specified by the manufacturer. The presence and the integrity of the insert sequence were confirmed by DNA sequence analysis. Sequencing was conducted with the same universal forward and reverse primers. Thereafter the plasmids harbouring the 16S rDNA sequence of *E. coli* were packaged into MS2 coliphages. To this end, a plasmid containing cDNA of MS2 bacteriophage was constructed. After digestion of this plasmid with BbvCI (New England Biolabs), the target was inserted downstream of the coat protein sequence of the MS2 (7, 8) and cloned into

pGEM-T easy vector. The system was induced with 1mM IPTG and bacteriophage particles were assembled. The phage-particles were fractionated on a CsCl gradient (5). The purified RNA was treated with RNases A and T1 and DNase I to assess its resistance to nucleases (5). Bacteriophage titer analysis showed that the armored RNA preparation contained $7x10^4$ copies of 16S rRNA/μL.

Reverse transcription-PCR sensitivity for 16S RNA standard

The RNA particles were used as a standard in a quantitative Real-Time PCR. For this purpose, serial dilutions of the armored RNA in TSM buffer (10 mM Tris (pH 7.0), 100 mM NaCl, 1 mM $MgCl_2$, 0.3% sodium azide and 0.1% gelatin) were made. RNA was isolated from 200 μL of each dilution with the same kit as described above. The RNA was eluted in a total volume of 50 μL. Twenty μL (2/5 of the extracted RNA) of this volume was used to produce cDNA. Ten μL cDNA (1/5) was used as template in Real-Time PCR. The PCR amplification (in 25 μL total volume) was performed in an ABI 7000 system (Applied Biosystems) as described in a previous study (6) with a universal primers (900 nM each) and probe (200 nM)(FAM-5'-CGTATTACCGCGGCTGCTGGCAC-3'-TAMRA) set and the TaqMan universal PCR Master Mix.

Quantification of DNA and RNA

To quantify the presence and the viability of bacteria in a molecular growth curve, samples from suspensions of *E. coli* grown in LB medium and taken at different stages of growth were analyzed. Total Nucleic Acid was extracted at each stage with the automated MagNA Pure system (Roche Diagnostics) from 200 μL duplicate samples according to the manufacturer's instructions. Nucleic acid was eluted in a total volume of 50 μL. Fifty μL was subjected to DNase I treatment using the DNA-free kit (Ambion Inc). The resulting RNA was assessed with gel electrophoresis and spectrophotometry. cDNA was generated as detailed above. Total ribosomal DNA and synthesized cDNA were amplified by Real-Time PCR, the armored 16S rRNA was used as a standard.

Effect of antibiotics on DNA/RNA content of *E. coli*

The effect of two antibiotics on the quantities of DNA and RNA detected during the molecular growth curve of *E. coli* was monitored. These antibiotics were chloramphenicol, a bacteriostatic agent, member of the miscellaneous family of antibiotics, and amikacin as a representative of the bactericidal aminoglycosides. *E. coli* (ATCC) was grown at 37°C in LB

medium to the logarithmic phase. The culture was then divided into 3 equal fractions; chloramphenicol (30 μg/mL) was added to the first and amikacin (30 μg/mL) to the second fraction. The third fraction remained untreated and served as control. The doses of antibiotics were chosen, based on the resistance breakpoints of the National Committee for Clinical Laboratory Standards (NCCls) guidelines. The bacteria were then incubated further at 37^{o}C until the decline (death) phase. During incubation samples were taken each 30 minutes. In each sample, 16S rDNA and 16S rRNA were quantitated by extraction as described above and Real-Time (rt)-PCR assays.

To assess the recovery of bacteria from the effect of antibiotics, the amount of 16S rDNA and 16S rRNA were determined after inoculation of the antibiotic-treated fractions into fresh LB medium with no drugs and further incubation for up to two hours.

Viability of *E. coli* in platelet concentrates

To determine the utility of the assay for detection of viable bacteria in clinical samples, DNA and RNA content of *E. coli* grown in human platelet concentrates (PCs) was determined. PCs were spiked with *E. coli* at an inoculum of 100 CFU/mL and monitored during a storage period of 7 days at 20 - 24^{o}C. On day 0 (day of spiking of PCs) 1, 2, 3, 6 and 7 samples 200 μL were analysed for the presence and viability of bacteria.

RESULTS

Sensitivity of the assay:

To quantify the amount of bacterial RNA in a sample, a stable RNA standard was needed. For this purpose, a fragment encoding 16S rRNA of *E. coli* was synthesized, cloned and packaged as armored RNA in MS2 coliphages.

The sensitivity of the rt-PCR was evaluated with limiting dilutions of the RNA standard. As shown in Fig.1 the assay was linear between $1.4x10^{4}$ and $1.4x10^{1}$ copies/reaction indicating that 14 copies of *E. coli* RNA per PCR

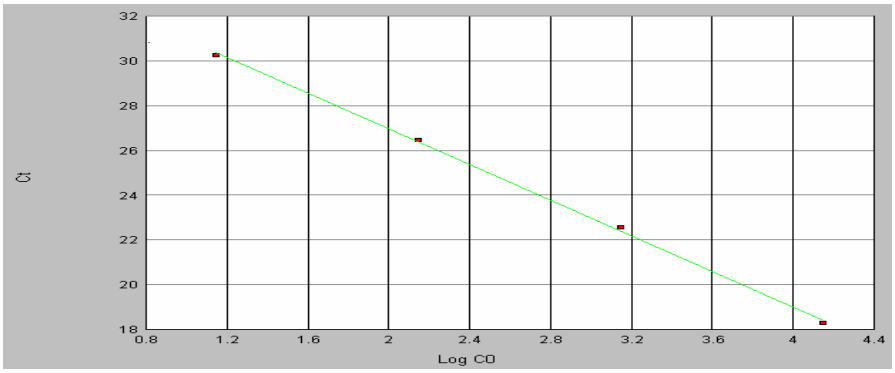


Fig.1: cDNA produced from serial dilutions representing $1.4x10^4$, $1.4x10^3$, $1.4x10^2$ and $1.4x10^1$ copies/reaction were used to determine the detection limit of the quantitative real-time rt-PCR for Armored 16S rRNA standard. The X-axis represents the starting copy number of the 16S rRNA expressed as log 16S rRNA/PCR reaction. The Y-axis represents the threshold cycle (C_T).

was the lower limit of detection. This corresponds to a detection limit of 70 copies in the starting volume in the extraction procedure. The same results were also obtained when the particles were first incubated at 70°C for 5 min to release the RNA from the phage (data not shown).

DNA/RNA content during growth of *E. coli* in LB medium and in PCs:

As depicted in Fig.2 the levels of DNA and RNA were dependent on the growth phase of *E. coli*: the amounts of both nucleic acids increased during growth to achieve a maximum at the end of the log-phase (Fig. 2). After the stationary phase the amounts decreased to a minimum (or undetectable levels). Since DNA originates from both viable and non-viable bacteria this nucleic acid was detectable in all stages of the growth curve, whereas 16S rRNA was detected only during the logarithmic and the stationary phase, but disappeared completely during the last phase (after 5 hours) of the growth curve, while in this phase DNA was still detectable. This implies that the bacteria were not viable any more. The results were reproducible in independent assays. Hence, these preliminary data demonstrate that determination of the DNA and RNA content provides information about the absence or presence and the state of viability of bacteria.

A correlation between viability and 16S rRNA content was also observed when *E. coli* was cultured in PCs (Fig. 3). RNA increased during growth to achieve a maximum at day 2 (day 0 is the day of spiking) of storage.

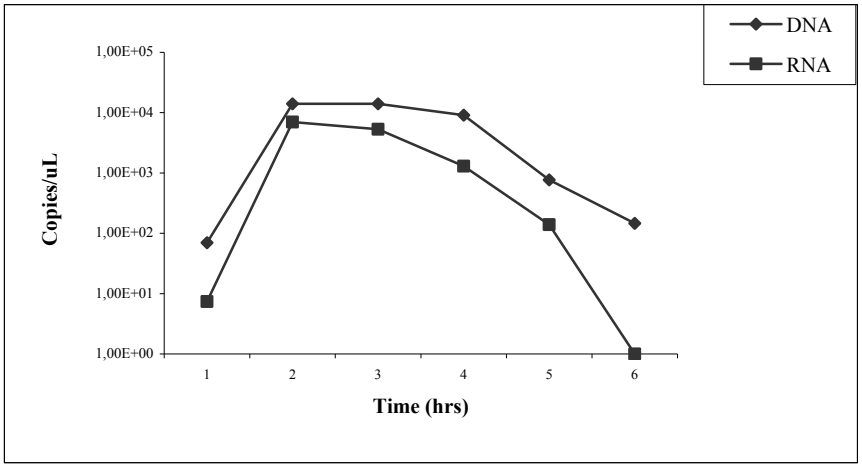


Figure 2: Quantitation of 16S ribosomal DNA and RNA during the growth curve of *E. coli* in LB medium at 37°C. The X-axis reflects the time points when the samples were taken. The number of copies/µL is represented on the Y-axis.

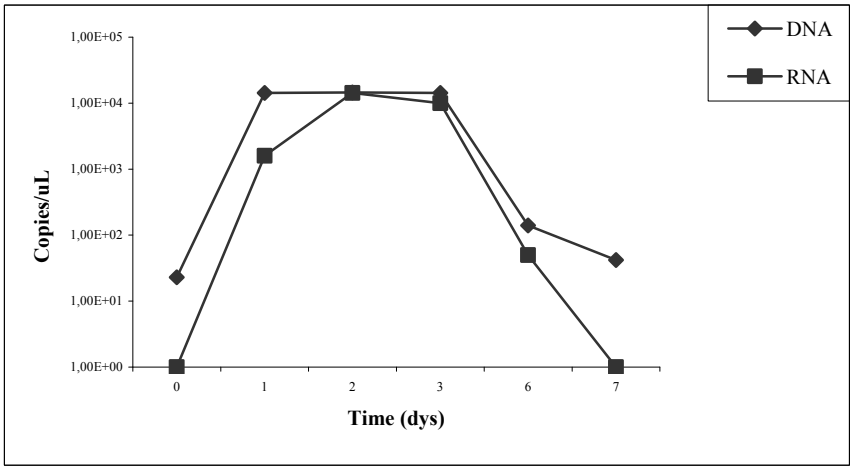


Figure 3: Quantitation of 16S ribosomal DNA and RNA during growth of *E. coli* in platelet concentrates. PCs were spiked with a suspension of *E. coli* at an inoculum of 100CFU/mL. PCs were stored at room temperature for up to seven days. The amounts of DNA and RNA were determined directly after spiking, on day 1, 2, 3, 6 and 7 of storage. The time points of sampling are reflected on the X-axis and the number of copies/µL is represented on the Y-axis.

Thereafter the RNA content diminished considerably and became undetectable on day 7 of storage. At this stage, DNA was still detectable. This suggests that although bacteria were present they were not viable.

Effect of antibiotics at the molecular level:

The effect of chloramphenicol and amikacin on the RNA content of *E. coli* is represented in Fig. 4. Addition of amikacin led to an immediate diminution of the number of RNA copies, compared to untreated culture. This amount persisted during one hour, thereafter no RNA was detectable any more. When bacteria were exposed to chloramphenicol during the logarithmic phase the content of 16S rRNA remained constant for two hours and then decreased to undetectable levels.

The DNA content was also affected: in comparison to the untreated samples, DNA declined with approximately 19 orders of magnitude after one hour exposure to the antibiotic. Thereafter the quantity of DNA remained relatively constant during three hours and declined thereafter.

When the treated cultures were inoculated in fresh LB broth without antibiotics, 16S rRNA became detectable again after two hours of incubaation only in cultures that had been exposed to the bacteriostatic antibiotic chloramphenicol. In contrast, in a culture of bacteria treated with amikacin in fresh broth no 16S rRNA could be detected. This suggests that bacteria recovered from the effect of chloramphenicol and started growing again, while the effect of amikacin was bactericidal.

With regard to the 16S rDNA content of the antibiotic-treated cultures, DNA was detected in both cultures: after amikacin treatment the DNA content did not change during incubaction in fresh broth, whereas after chloramphenicol treatment the detection of 16S rRNA was accompanied by an increase in the 16S rDNA content (Fig.5).

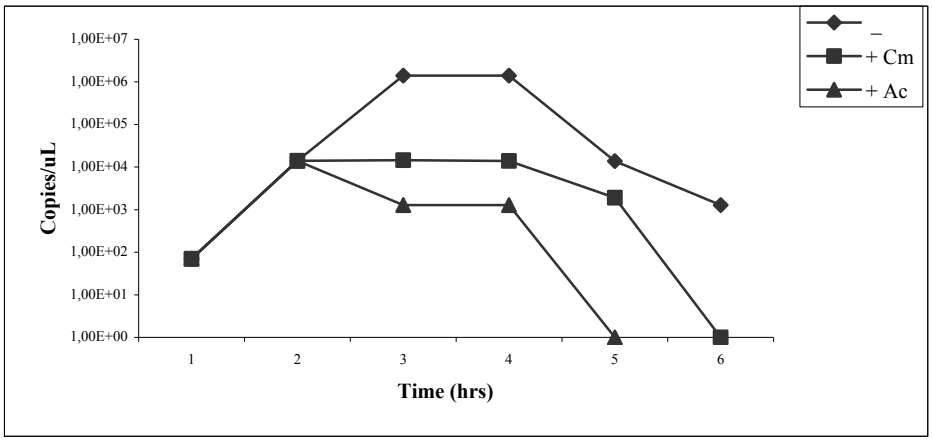


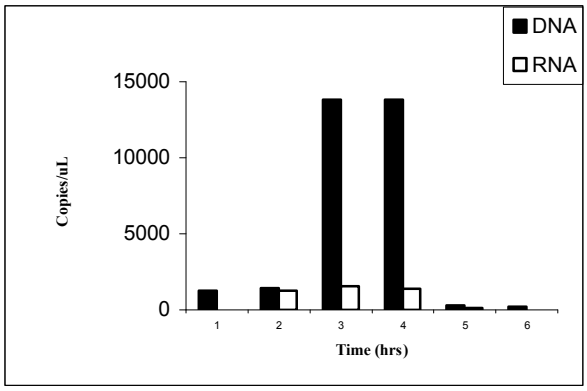
Figure 4: Effect of chloramphenicol and amikacin on the growth of *E. coli*. The plots represent the amount of RNA estimated by real-time PCR in untreated culture (-) and in cultures exposed to chloramphenicol (Cm)(30 µg/mL) or amikacin (Ac)(30 µg/mL). These antibiotics were added at time point 2. The sampling time is reflected on the X-axis and the number of copies/µL on the Y-axis.

DISCUSSION

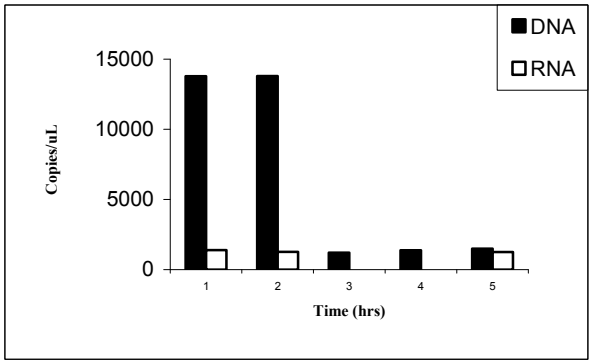
In the present study, the hypothesis that simultaneous determination of DNA and ribosomal RNA could provide an indication of the viability of bacteria present in a sample was tested. DNA was quantified by amplification of the 16S rDNA gene and comparison to a standard curve, generated by simultaneous determination of colony forming units. To quantify the amount of bacterial ribosomal RNA, a standard curve was also needed. To this end, a stable armored ribosomal RNA was devised and used as standard for quantitating RNA, as well as internal and inhibition control in the rt-PCR protocols.

The use of this standard allowed the monitoring of the growth of *E. coli* in LB medium by quantifying both DNA and RNA. These quantities corresponded well with the presence and viability of bacteria. Since RNA was detectable during the logarithmic phase of growth and disappeared during the decline phase, this nucleic acid can be used as indicator of viability. The DNA/RNA content can be employed to predict the metabolic state (living or dying) of bacteria.

A. Untreated



B. + Chloramphenicol (30 µg/mL)



C. + Amikacin (30 µg/mL)

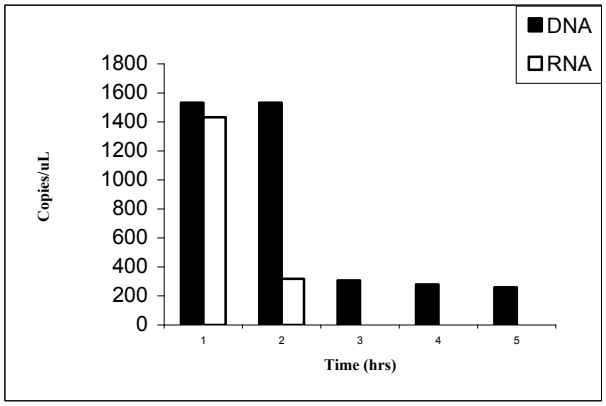


Figure 5: Assessment of the presence of DNA and RNA of *E. coli* grown in LB medium at 37^{o}C (A), upon exposure and recovery from antibiotics. During the logarithmic phase of growth the cell culture was divided into three fractions. The first fraction remained untreated (A), chloramphenicol was added to the second fraction (B) and amikacin to the third fraction (C). The antibiotics were added at time point 2 of fraction (A). After point 3 of B and C the cultures were transferred to fresh LB medium with no antibiotics.

The assay was further exploited to monitor the effect of two distinct antibiotics on the growth curve of *E. coli*. A correlation between viability and the number of 16S rRNA copies was observed: addition of the bacteriostatic antibiotic chloramphenicol resulted in considerable decrease of number of copies of 16S ribosomal RNA, to undetectable levels after 4 hours of incubation, indicating a loss of viability. A similar effect was also achieved when amikacin was added. However, with this bactericidal agent the decrease was more rapid. This might be due to the difference in effect on the turnover of ribosomal RNA between these antibiotics, sincechloramphenicol is bacteriostatic while amikacin is bactericidal. Chloramphenicol inhibits bacterial growth without killing; as a result the synthesis of new RNA is slow while degradation is rapid. Amikacin leads to the death of bacteria, which results in the absence of the synthesis of RNA and rapid degradation of already present nucleic acid.
In addition the recovery of the synthesis of 16S rRNA from the effect of chloramphenicol was demonstrated after inoculation of bacteria in broth with no drugs. As expected, no recovery from the effect of amikacin was detected although detectable levels of 16S rDNA were present. This indicates that the presence of DNA is independent of the viability of bacteria as with this assay DNA can be detected in both viable and non-viable bacteria.

To demonstrate the utility of this approach, the presence of viable bacteria was also studied in PCs as clinical material. Transfusion of these blood therapeutics can be associated with transmission of bacterial infections. Storage of PCs at room temperature for up to seven days facilitates proliferation of bacteria. To ensure safety of transfusion, PCs have to be sterile before administration. Recently, a method based on detection of 16S rDNA was developed and used to screen PCs for bacterial contamination. However, this method cannot distinguish between living and dead bacteria. By determining the DNA and RNA content, it was possible to demonstrate the presence of *E. coli* in PCs and its state of viability. This approach can therefore be applied to estimate the clinical significance of the presence of bacterial DNA in blood products and improve the management of PCs transfusions.

The universal nature of the primers and the probe set used in this real-time PCR renders it useful to determine the viability of noncultivable bacteria in clinical samples. Quantitation of DNA and RNA could also be used to monitor the effects of antibiotics during treatments of infections with bacterial pathogens. This specific application of this real-time PCR merits further investigation and may lead to new insights in optimization of antimicrobial therapy.

Finally, the RNA standard is suitable for all real-time amplification protocols. Since the armored RNA standard withstands nucleases degradation, it can be added to clinical samples prior to nucleic acid extraction to monitor the whole process of isolation, reverse transcription amplification and detection.

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Chapter 9

Summary and general discussion

Samenvatting

Résumé

Summary and general discussion

Platelets, the smallest anucleated cells of blood, are important in hemostasis (cessation of bleeding). In the blood stream, platelets circulate as disc-shaped cell fragments. Following external damage to blood vessels, they adhere to subendothelial substances such as collagen. Upon exposure to collagen, platelets become activated and change from discoidal to spheres. This shape transition is followed by forming of aggregates or haemostatic plugs, which are indispensable for stopping bleeding.

Platelets are used in the management of patients suffering from bleeding disorders, for example thrombocytopenic patients, patients receiving chemotherapy or patients submitted to bone marrow or stem cells transplantation.

Platelets are issued as concentrates. Platelets concentrates (PCs) are routinely prepared from donated whole blood. Once collected in an integrated system of plastic bags, whole blood can be converted to its components (i.e. plasma, platelets, and red blood cells) by application of two step-centrifugation. Dependent on the speed and the length of each step PCs can be obtained using the platelet-rich plasma (PRP) or the buffy coat (BC) method. After preparation, PCs are stored at 20 - 24 ^{o}C for a shelf life of 5 to 7 days.

PCs, stored at room temperature, are attractive for growth of bacteria. Consequently, contamination with bacteria may turn transfusion of PCs from a life-saving into a life-threatening therapy.

To date, bacterial contamination of PCs is acknowledged as the most frequent transfusion-associated infectious risk. To reduce and prevent this risk from occurring, methods to limit and detect bacterial contamination of PCs prior to transfusion are required.

Over the last decades, major improvements have been made in the development of detection strategies. The available methods vary from simple to very sophisticated. A brief synopsis of existing approaches to assess the presence of bacteria in PCs was given in ***Chapter 1***. As detailed in the general introduction each approach has advantages and disadvantages and an 'ideal' method is as yet lacking.

Automated blood culture methods are being used currently in several countries. In some of these the implementation of this culture system in routine use has led to the extension of the shelf life of PCs from 5 to 7 days. For automated culturing of PCs different protocols are currently in use. In some countries culturing is performed shortly after preparation of blood components, whereas in others it is done only for platelets that have not been transfused by

day 3 of storage. In addition, some countries maintain a quarantine period of 24-48 hours after culturing, before issuing to hospitals.

In The Netherlands a BacT/Alert automated culture system is incorporated into routine practice to screen all PCs for bacterial contamination since November 2001. An aliquot of 5-10 mL PCs, prepared following the BC-method, is used to inoculate both culture bottles of the BacT/Alert. Inoculation is carried out within 3 hours of preparation of PCs (22-24 h after whole blood collection or within 12 h of apheresis). The culture bottles are incubated until a positive signal is detected or for up to 7 days if they remain negative. During incubation PCs are issued to hospitals as 'negative to date' (i.e. at the time of issue of PCs to hospitals, the culture was negative). When a positive signal is detected, the involved culture bottles, the sample bag (with PCs used to inoculate the bottles) and the original PC (if available) are sent to a microbiology laboratory for confirmation and identification of contaminants. A PC is deemed contaminated when a microorganism is identified in the culture bottles. The implicated PCs and related blood products are then returned to the blood bank via a recall procedure.

Although automated culturing has proven to be sensitive and robust in detection of bacterially contaminated PCs, it requires 24-48 hours to yield (positive) results for most organisms. During this incubation time PCs might be already issued and be administered to a recipient. The work described in this thesis was, therefore, devoted to develop an alternative tool that overcomes the limitations of the current detection strategy to screen PCs for bacterial contamination.

Based on real-time quantitative PCR technology, a broad-range 16S rDNA PCR assay was developed and adapted to detect bacterial DNA in PCs. The performance of this PCR assay was dependent on two critical factors: the efficiency of the method used to extract DNA from PCs and the amplification procedure. To achieve optimal sensitivity with this assay a convenient method to prepare template DNA from PCs was required. Two isolations methods were assessed and compared as described in ***Chapter 2***. Since PCs have a short shelf life (5-7 days) a rapid assay was desirable. For this reason, these 2 extraction methods were mainly judged according to their sensitivity and rapidity to obtain results. Both NucliSens extraction method as automated MagNA Pure LC instrument proved to have a detection limit of 1 colony forming unit (CFU) equivalent/PCR. Given the higher throughput and the shorter

processing time, the fully automated MagNA Pure system was preferred. Furthermore, this system provides a standardized and reproducible method for nucleic acid isolation, which is amenable for routine practices.

During the course of the analytical validation of the PCR assay, a major problem was encountered. The problem manifested in the occurrence of false-positive results caused by the amplification of contaminating DNA present in the PCR reagents (but not in the actual sample of PCs to be analyzed). Owing to this common limitation of PCR assays, efforts made by several investigators in the past to develop a PCR-based detection method were abrogated.

This background contaminating DNA was effectively reduced by following two strategies. These were described in Chapter 2 and their effect on the sensitivity of the PCR assay was also discussed.

Before amplification, bacterial DNA must be extracted from the specimen to be analyzed. This extraction procedure is the first and most critical step in real-time PCR assays. Therefore, effective methods of DNA extraction are warranted in order to prevent variation in (real-time) PCR results. To monitor the efficiency of nucleic acid extraction with the MagNA Pure, an internal control was included in each extraction. This control amplified a conserved region of the human *HLA DQα* gene. As described in ***Chapter 3***, a set of primers and probe targeting a highly conserved region of the generic allelic group of HLA-DQA1 locus was first designed. The real-time amplification of this locus was then validated with an array of samples of PCs. Since each white blood cell (WBC) contains 2 copies of the target gene, the HLA-DQA1 could be extracted and amplified from the same sample of PCs. External addition of the control to PCs specimen was not needed. This is especially beneficial in reducing the amount of clinical material used to isolate DNA and hence the processing time and the corresponding costs. Furthermore with this control the risk of the introduction of contamination into the samples to be tested is reduced. Moreover amplification and detection of both this internal control and bacterial-specific DNA can be accomplished in the same reaction.

Because the HLA-DQA1 was reproducibly and consistently amplified in all tested samples, the assay was also adapted to count residual WBCs in filtered PCs. As detailed in chapter 3 reduction of these cells is necessary to prevent a variety of transfusion complications. Nowadays, several strategies have been institutioned in blood centers to estimate the number

of WBCs in filtered PCs as part of the QC of PCs. Compared to flow cytometry (current gold standard); the assay was shown to be sensitive, accurate and rapid. These characteristics make it very suitable for routine monitoring of residual WBCs in PCs.

Thus, performing this study a double purpose was attained: the universal nature of this HLA-DQA1 assay is suitable to monitor efficiency of automated nucleic acid extraction from blood samples in routine diagnostics laboratories, and also for blood centers for estimating the number of WBCs in PCs.

Once all critical aspects of the 16S rDNA real-time PCR were optimized, and the assay was complete a clinical validation was commenced. Unfortunately preliminary results of screening PCs for bacterial contamination revealed the rise of false-positive results yet again. This time contamination came from an unexpected quarter: the source was tracked to contaminating DNA present in the commercially available reagents of the MagNA Pure nucleic acid extraction kit. Considering the nature of bacteria found in the reagents, it was conceivable that contamination originates from the distilling systems of the manufacturer. Elimination of this contaminating DNA has proved very difficult. Nevertheless, a strategy reported in ***Chapter 4*** was successfully applied to remove this reagent-derived contamination while the sensitivity of the assay was maintained.

As discussed above to achieve optimal sensitivity of the PCR assay, standardized DNA extraction procedures, with high-quality nucleic acid purification are needed. So far, the 16S rDNA PCR in combination with the MagNA Pure automated DNA extraction seemed to meet this requirement.

To assess the applicability of this method for routine use in blood centers, the PCR assay was validated in an extensive survey of 2,146 routinely produced PCs. This was done in conjunction with the BacT/Alert automated culture system, which is regarded as the gold standard. The findings of the assay as reported in ***Chapter 5*** were in complete agreement with those obtained with the BacT/Alert. The PCR assay showed the same sensitivity and specificity as culture, with the advantage of a shorter turnaround time (4 hours). Subsequently, PCs can be tested before release or the day they are transfused. Unlike culture, detection with real-time PCR does not depend on the growth kinetics of bacteria. This feature permits the extension of the storage time of PCs from 5 to 7 days without compromising the risk of transfusing contaminated units. However, it is worthwhile to mention that the PCR detects both viable and non-viable microorganisms. Up to now the clinical significance of

receiving dead bacteria is unclear; the use of this approach may result in unnecessary wastage of a limited blood resource. Taken together, combining automated DNA extraction and sample amplification with real-time PCR has made possible the development of an assay that was shown to be rapid and sensitive. This PCR assay offers an alternative to the BacT/Alert system for the screening of PCs. Before implementing such an assay in routine use it might be essential to conduct a cost-benefit analysis.

The amount of bacteria (when present) changes with storage time of PCs. As a result confirmation of contamination becomes complicated. If a sample is taken on day 1 after preparation with low levels of bacteria, most detection systems (no matter how sensitive) would fail to assess their presence. On the other hand if sampling is carried out from day 2 of storage or longer, the majority of systems regardless of their sensitivity will indicate the presence of bacteria. Accordingly the sensitivity of detection systems is related to time when a sample was taken for testing. Another important facet, when testing for bacterial contamination, is the volume of sample used for culture and the probability of the presence of a bacterium in the sample taken from the PCs bag.

To determine the optimal time for sampling PCs when real-time quantitative PCR is employed to detect bacterial contamination, an evaluation study (***Chapter 6***) was carried out with the BacT/Alert as reference method. PCs were spiked with suspensions of *E. coli*, *B. cereus*, *S. epidermidis*, *P. aeruginosa* and *P. acnes* to 1, 10 and 100 CFU/mL. With the PCR assay all spiked bacterial species (at different concentrations) could be reliably detected one day after inoculation and preparation of PCs regardless of the size of the inoculum. Whereas in the BacT/Alert all concentrations required an incubation time of at least 20.1 hours. This indicates the efficacy of detection with PCR and offers the possibility of removing contaminated PCs prior to issue.

Currently, in the routine of the blood bank PCs are sampled within 3 hours after preparation for culturing in the BacT/Alert and issued as negative to date. Based on the findings of this study, it would be recommended to sample the PCs at least one day after preparation when using either BacT/Alert or PCR to guarantee detection of all possibly contaminated units. This may contribute to further improving the safety of PCs supply and limiting the consequences of the policy of issuing as negative to date.

Summarizing (Chapter 2 through 6), the PCR assay was shown to meet the requirements of the ideal detection methods defined in the introduction (Chapter 1). The findings demonstrated a sensitivity of 1 CFU/mL (50 CFU/mL) of the PCR assay, which is an improvement of the currently used methods. The results are provided within 4 hours. The assay can be performed one day after preparation of PCs and can be used before release of PCs to hospitals or when carried out in the hospital immediately before they are administered to patients.

Propionibacterium acnes is implicated in most cases of bacterial contamination of PCs. It is generally thought that this bacterium originates from skin flora. **Chapter 7** was confined to determine the source of contamination using amplified fragment length polymorphism (AFLP) analysis. *Propionibacterium* isolates from PCs and RBCs from the same donations were analyzed. The isolates could be classified into 3 groups: group 1 including 45% of the isolates belonging to the surface of the skin flora, group 2 and group 3 (55% together) different from each other and from group 1 originating from another habitat. These 2 groups could be subspecies *of P. acnes*. However evidence could not be obtained from the results. Since only 45% of the isolates were shown to belong to the surface of the skin flora, it should be apparent to reconsider the efficacy of introducing measures such as improving skin cleansing methods and diverting. Other studies are needed to determine the molecular relatedness of different species of bacteria commonly found in blood products. Exploiting the AFLP technique for further typing of bacteria found in whole blood (e.g. before and after discarding the first aliquots), plasma, PCs, and RBCs would presumably provide more insights into the nature and possibly the source of contamination. Adequate measures would be then taken by blood centers to allay this problem.

Real-time PCR is a convenient way to detect pathogens. It allows the simultaneous amplification, detection and quantification of target nucleic acid. Nevertheless, the 16S rDNA PCR assay has a limitation: it does not distinguish between living and dead organisms. This distinction might be important to study the clinical significance of receiving blood components contaminated with bacteria. Furthermore, it would be possible to study the survival of bacteria (particularly at low initial levels) during storage of PCs. Based on quantitation of DNA and RNA a method was developed and adapted to study the presence

and viability of *E. coli* in both broth as well as PCs (**Chapter 8**). For quantitation an armored RNA standard, with 16S rRNA of *E. coli* as target, was developed and employed in the assay. The standard was resistant to degradation by nucleases. Applying this, it was possible to determine the RNA/DNA content to predict the state (viable or non-viable) of viability of bacteria. The effect of 2 distinct antibiotics on the molecular growth curve of *E. coli* was also monitored. With this new approach a twofold objective was achieved: (i) the method can be used to monitor the effects of antibiotics during treatments of infections with bacterial pathogens. This may contribute to monitoring the efficacy of antimicrobial therapy in patients, which currently relies on cell culture techniques (ii) the standard may also be suitable to be used as internal and inhibition control in real-time quantitative RT-PCR assays. To improve the versatility, this approach may be enlarged with standards targeting different pathogens and tests in different clinical materials.
However, due to the high costs at present, a widespread use of this assay in routine diagnostics laboratories may be restricted.

Follow up studies

Developing a suitable method to detect bacteria in PCs for use under routine blood banking conditions is challenging, because the method must not only be sensitive, specific and rapid but must also provide logistic and economic efficiency. Next to the high costs, the time to obtain results of the current developed 16S rDNA real-time PCR may still be long. An initial cost-benefit analysis may therefore not be favorable. Nevertheless the assay forms an improvement to current detection methods, at least to the automated culturing with regard to sensitivity and duration. Furthermore the potential to automate and standardize the critical steps in the amplification procedures of the assay, the manufacturing of DNA-free reagents, and the use in a large scale may presumably accelerate the time to obtain results and reduce the costs of the assay.
In addition, the universal nature of detection of the assay may be suitable for generalized bacterial screening of other blood components (e.g. RBCs and plasma). Therefore it is necessary to adapt and optimize detection in RBCs and plasma with this broad-range PCR.

The approach developed to study the viability of bacteria, while technically demanding for routine use, may be simplified and standardized to afford a potentially convenient method for

distinction between viable and non-viable bacteria when present in PCs. Besides, this approach offers the possibility to investigate the bactericidal effect of plasma on bacteria contaminating PCs, in comparison with other platelet additive solutions (used as alternatives of plasma).

The differentiation of viable and non-viable bacteria could be applicable to several clinical materials and useful in the diagnosis and treatment of infections. The assay has the potential to allow the monitoring of the effect of antibiotics. This would prevent the unnecessary use of antibiotics, a better management of patients and finally a reduction of health costs. However, further extensive studies with other clinical materials and a wide array of antibiotics are needed to determine the clinical utility of this approach.

The safety of blood components supply is a major public health concern. To remedy the risk of transfusion-transmitted bacterial infections, most attempts have been focused on pre-transfusion detection of contamination. Several detection techniques have shown value, however, it is virtually impossible to guarantee 'bacteria-free' platelets supply. Therefore it would be worthy to combine different approaches contemplating current technologies. Attention should be given to the prevention of contamination by investigating for example other conditions to store PCs:

Platelets are stored at room temperature for a regulated short storage period of maximal 7 days. Next to the risk of bacterial contamination, platelets undergo during storage a number of changes that adversely affect their viability and hemostatic function (often referred as platelet storage lesion) after transfusion. Why platelets lose viability during storage has preoccupied several investigators in the past. It has been postulated that platelet possesses an intact mechanism of apoptosis. This mechanism is somehow enhanced during storage. Hitherto, must studies of apoptosis have been performed in nucleated cells, in which the nucleus is involved in the apoptotic process. However, 7 years ago Vanags et al. [(1)] reported on the existence of such a process in platelets (anucleated cells). Recently, Bertino et al [(2)]. speculated that apoptosis is accelerated during storage at 37^{o}C. It was also suggested that this process was responsible for the rapid decline in platelet cellular viability.

In nucleate cells, it is well known that apoptosis includes two main pathways: one is triggered through death receptors, and the other is initiated by mitochondrial changes. Both pathways are not clearly understood, and involve the participation of a cascade of molecules. Fas is one of the most important molecules in death receptor-mediated pathway. The Bcl-2 family

proteins are involved in the mitochondrial-mediated pathway. Alterations in the ratio between pro-apoptotic (e.g. Bak, Bax, Bid) and anti-apoptotic (e.g. Bcl-$_{XL}$) proteins are thought to be responsible for regulation of the apoptotic process [3,4].

During a pilot study (not incorporated in the chapters of this thesis) Fas and Bcl-$_{XL}$ were employed as markers to study apoptosis during storage of PCs under standard blood banking conditions (i.e. room temperature for up to 7 days), and at 4^{o}C and 37^{o}C. Fas and Bcl-$_{XL}$ expression was studied using Pre-Developed Assay reagents (PDARs) in a reverse-transcription Real-Time PCR. During storage the parameters to assess the viability of PCs (e.g. pH measurements, gas exchange and glucose metabolism) were monitored.

Initially, the critical steps of the assay including the isolation of RNA from PCs, extraction and amplification efficiencies were optimized. Hypoxanthine ribosyl transferase (HPRT) was used as an endogene control for gene expression.

The results demonstrated no significant differences in the expression of Bcl-$_{XL}$ over time and under different storage temperatures. On the other hand, a decrease in the expression of this marker was observed after 7 days at both room temperature and 37^{o}C. These observations were in agreement with what was found by Bertino and coworkers [2].

The expression of Fas was not found in routinely prepared PCs stored at room temperature, at 4^{o}C or at 37^{o}C for up to 7 days. The expression was only detected in PCs stored at 37^{o}C in the presence of anisomycin (an apoptosis inducer), although no alterations were registered in the loss of viability. However, the significance of these preliminary findings requires further investigations and extensive studies with different markers to establish a model for platelet apoptosis. Understanding apoptotic processes responsible for platelet storage lesion might provide insights into possibilities for inhibition of this phenomenon. This could be done by taking advantage of the availability of new advanced technologies such as RNA-interference (RNAi). Silencing genes involved in the decision to undergo apoptosis might result in extending the storage of platelets.

Studying apoptosis in relation to loss of viability under different storage conditions remain to be carried out. These studies should be also aimed at reducing bacterial contamination while prolonging platelet shelf life. To achieve this, storage at lower temperatures (e.g. 4^{o}C) seems more favorable. Storage at this temperature would be expected prevent bacteria from proliferating. Hoffmeister and collegues [5] described recently in a breakthrough study a simple in vitro method to maintain survival of platelets while stored at 4^{o}C. It is well known

that cooled platelets are cleared rapidly after transfusion. This is due to the fact that on the surface of cooled platelets clusters of proteins form a receptor complex. This latter is recognized and phagocytozed. This process could be prevented by inducing glycosylation by adding Uridine Diphosphate-Galactose (UDP-G) before or after storage at $4^{o}C$ [(5)].

These promising developments may offer new perspectives in managing both the issue of the short shelf life of platelets and the concern of bacterial contamination in the foreseeable future.

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Samenvatting

Samenvatting

Bloedplaatjes, ook wel trombocyten genoemd, zijn de kleinste ‘cellen’ in het bloed. Deze celfragmenten van voorlopercellen uit het beenmerg (megakaryocyten) zijn onontbeerlijk voor het in gang zetten van de hemostase (stoppen van bloedingen).

In de bloedbaan circuleren bloedplaatjes als schijf-vormige celfragmenten. Bij beschadiging van de bloedvatwand vormen zij uitsteeksels, kleven aan elkaar (aggregatie) en aan de bloedvatwand (adhesie) en storten biochemische substanties uit waardoor stollingsfactoren worden geactiveerd. Eerst vormt zich een prop van bloedplaatjes die het gat voorlopig sluit, later dicht een stolsel het gat definitief.

Bloedplaatjes worden toegediend aan patiënten met een ernstig tekort aan trombocyten (trombocytopenie) of aan patiënten met niet goed werkende bloedplaatjes en een verhoogd risico op bloedingen bijvoorbeeld tijdens een operatie, transplantatie of tijdens een behandeling met chemotherapie.

Bloedplaatjes worden als concentraten getransfundeerd. Deze plaatjesconcentraten (PC) worden gemaakt van vol bloed. Na het doneren van ½ liter bloed door een gezonde vrijwilliger in een steriel systeem van bloedzakken, wordt het bloed gescheiden d.m.v centrifugatie in drie verschillende componenten: plasma, buffycoat en rode bloedcellen. In de buffycoat, de laag tussen plasma en rode bloedcellen, zijn de meeste bloedplaatjes en witte bloedcellen aanwezig. Voor een transfusie aan een volwassene worden vijf buffycoats samengevoegd met een eenheid plasma, dit mengsel wordt nogmaals gecentrifugeerd om een plaatjesconcentraat te krijgen.

Na bereiding kunnen PC maximaal 7 dagen schuddend bij kamertemperatuur bewaard worden. Deze bewaarcondities maken de PC geschikt voor de groei van bacteriën. Transfusie van PC waarin bacteriën aanwezig zijn kan ernstige bloedvergiftiging (sepsis) bij ontvangers veroorzaken met soms dodelijke afloop. Om dit risico te verkleinen worden tijdens de afname en bereiding allerlei maatregelen genomen om binnendringen van bacteriën te voorkomen. Omdat die maatregelen niet altijd afdoende zijn, zijn methoden (technieken) nodig die met grote gevoeligheid en snelheid de aanwezigheid van bacteriën in PC kunnen aantonen vóór transfusie.

In de afgelopen jaren zijn diverse methoden ontwikkeld en geïmplementeerd. Echter een ideale methode is nog niet beschikbaar.

In Nederland is sinds 1 november 2001 bacteriële screening van alle plaatjesconcentraten ingevoerd. Deze screening wordt gedaan met behulp van een geautomatiseerd kweeksysteem (BacT/Alert). Hiertoe wordt een PC monster van 5-10 mL geïnoculeerd in twee kweekflesjes van de BacT/Alert. Het ene flesje is bedoeld om bacteriën die zuurstof nodig hebben om te groeien aan te tonen en het andere om bacteriën die zonder zuurstof groeien aan te tonen. Het kweken duurt max. 7 dagen of totdat een positief signaal is gedetecteerd. PC worden uitgegeven op basis van "negative to date" criterium. Dit wil zeggen dat op het moment van uitgifte van de PC van de bloedbank aan het ziekenhuis, de kweek negatief is.
Bij een positief resultaat van de kweek, worden PC en alle afgeleide bloedproducten (plasma en rode bloedcellen) van de betrokken donaties geblokkeerd of teruggeroepen voor respectievelijk, kweken, bevestiging (confirmatie) en identificatie van de gevonden bacteriën.
Hoewel met de BacT/Alert de aanwezigheid van bacteriën opgespoord kan worden, is het resultaat meestal pas na 24-48 uur beschikbaar. Dit heeft als nadeel dat tijdens de incubatietijd, PC al uitgegeven en mogelijk getransfundeerd kunnen zijn. Het voornaamste doel van dit onderzoek was dan ook het ontwikkelen van een alternatieve methode die de beperking van het huidige kweeksysteem ondervangt.

Zoals beschreven in hoofdstuk 2 werd een assay, gebaseerd op de real-time 16S rDNA PCR, ontwikkeld en geoptimaliseerd om bacterieel DNA in PC te detecteren. De eerste en tevens de belangrijkste stap in deze assay was het isoleren van het nucleïnezuur (DNA) uit het gewenste PC monster. Om een optimale gevoeligheid met deze assay te verkrijgen werden twee verschillende methoden om DNA te isoleren uit PC vergeleken. Met zowel de Nuclisens als de MagNA Pure methode werd een detectie limiet van 1 CFU/PCR bereikt. Rekening houdend met de bewerkingstijd, standaardisatie en reproduceerbaarheid, werd de voorkeur gegeven aan het volledig geautomatiseerd MagNA Pure systeem als het meest geschikte systeem voor isolatie van bacterieel DNA uit PC in routine.
De gevoeligheid van de PCR assay kan beïnvloed worden door het ontstaan van vals-positieve resultaten als gevolg van vermenigvuldiging (amplificatie) van contaminerend DNA aanwezig in PCR reagentia. Dit probleem deed zich voor tijdens de analytische validatie van de test. Om deze achtergrond contaminatie te reduceren werden twee strategieën gevolgd. Het effect van deze methoden op de gevoeligheid van de test is ook beschreven in Hfd 2.

De 16S rDNA PCR assay bestaat uit twee belangrijke stappen: de extractie van het nucleïne zuur uit het klinische materiaal gevolgd door de amplificatie van de gewenste target DNA sequentie. Om contaminatie in (real-time) PCR resultaten te voorkomen, is een efficiënte methode om DNA te isoleren vereist. De efficiëntie van extractie van DNA kan gemonitored worden door een isolatiecontrole tijdens het extractieproces mee te nemen. In Hfd 3 is het gebruik van de amplificatie van het HLADQα gen als isolatiecontrole beschreven. Dit gen is aanwezig in 2 kopieën in witte bloedcellen (WBC). Door gebruik te maken van een geconserveerd gedeelte van het HLA-DQA1 locus, kan de integriteit van het extractiesysteem getest worden. De test was reproduceerbaar en bleek ook geschikt te zijn als methode om het restant aantal WBC in PC waaruit leukocyten verwijderd zijn, te tellen. Omdat WBC in bloedproducten schadelijke effecten kunnen hebben voor de ontvangers, worden zij verwijderd tijdens de bereiding. Het restant aantal WBC moet minder dan 1 x 10^6 zijn. Een vergelijkende studie tussen de real-time HLA-DQA1 PCR en WBC-telling met flow cytometrie (huidige gouden standaard) heeft laten zien dat de assay gevoelig, accuraat en snel is. Deze eigenschappen maken de HLA-DQA1 PCR test zeer geschikt om gebruikt te worden in de routine van PC screening op restant aantal WBC.
Samenvattend kan de test twee doelen dienen: enerzijds kan het gebruikt worden als extractiecontrole van geautomatiseerde systemen uit bloed(producten), anderzijds als test om het restant aantal WBC in PC in bloedbanken te bepalen.

Na het optimaliseren van alle stappen, was de 16S rDNA assay geschikt om gebruikt te worden om PC, in routine gemaakt, te screenen op de aanwezigheid van bacteriële contaminatie. Echter tijdens de eerste studies ontstond een probleem van vals-positieve resultaten. De bron was mogelijk het waterzuiveringsysteem van de fabrikant die de reagentia voor de extractie van DNA leverde. Reductie van deze contaminatie werd succesvol opgelost door de methoden beschreven in Hfd 4.
De resultaten van de validatie van de PCR assay zijn beschreven in Hfd 5. Hierbij werden 2.146 PC getest. Bacteriële screening werd gedaan met zowel PCR als met de BacT/Alert. De bevindingen van de PCR test waren in complete overeenstemming met die van het kweeksysteem m.b.t de gevoeligheid en specificiteit. De PCR assay heeft het voordeel van een korte opwerkingstijd (4 uur). Hiermee is het mogelijk om PC te testen vóór uitgifte of vlak vóór transfusie (in het ziekenhuis). Een belangrijk verschil tussen beide methoden, is dat

de PCR DNA detecteert dat afkomstig is van zowel levende als niet-levende bacteriën. Dit zou kunnen leiden tot onnodige verspilling (vernietigen) van schaarse bloedproducten. Alvorens besloten kan worden om deze methode te implementeren wordt daarom aanbevolen om een kosten-baten analyse uit te voeren.

De gevoeligheid van een systeem om bacteriën te detecteren is verbonden aan twee factoren,
i) het tijdstip van monstername: Tijdens de opslag van PC kan het aantal bacteriën (indien aanwezig) veranderen. Hierdoor wordt confirmatie ingewikkeld. Met andere woorden wanneer een monster wordt genomen op de dag van bereiding (dag 1) zal het merendeel van de detectiesystemen (hoe gevoelig dan ook) de aanwezigheid van bacteriën niet detecteren indien het aantal zeer laag is. Echter, wanneer bemonstering van PC plaats vindt vanaf dag 2 (ongeveer 24 uur na bereiding), zal de meerderheid van systemen de aanwezigheid van bacteriën aantonen, ongeacht de gevoeligheid.
ii) het volume van het genomen monster voor screening en de kans dat een bacterie aanwezig is in dit genomen monster.
Om de optimale tijd te bepalen voor monstername van PC voor bacteriële screening werd een evaluatie van de PCR assay gedaan (Hfd 6). Hierbij werden PC gespiked met suspensies van *E. coli, B. cereus*, *S. epidermidis*, *P. aeruginosa* en *P. acnes* in (eind)concentraties van 1, 10 en 100 CFU/mL. Met de PCR assay werden alle bacteriële species gedetecteerd binnen 4 uur, in monsters die genomen waren ongeveer 24 uur na inoculatie en bereiding onafhankelijk van de grootte van het inoculum. Daarentegen vereiste de detectie van bacteriën in de BacT/Alert een incubatie tijd van minstens 20.1 uur.
In de routine van de bloedbank, geschiedt bemonstering van PC binnen 3 uur na bereiding voor het kweken in de BacT/Alert. Op basis van de resultaten van deze studie verdient het aanbeveling om de monstername een dag na bereiding uit te voeren. Wanneer beide methoden worden gebruikt om mogelijk gecontamineerd PC op te sporen en zo de veiligheid van transfusies van trombocyten te verbeteren, geeft de PCR het snelste een resultaat.
Samenvattend, de PCR test voldoet aan de eisen van een ideale detectie methode als gedefinieerd in de introductie (Hfd 1). De assay heeft een gevoeligheid van 1 CFU/PCR (50 CFU/mL), en is een verbetering van de huidige kweekmethode. De resultaten zijn binnen 4 uur beschikbaar. De test kan uitgevoerd worden 1 dag na bereiding van PC. Het kan gebruikt worden vóór uitgifte of vlak voor toediening aan patiënten in het ziekenhuis.

Om onderscheid te maken tussen levende en dode bacteriën met de real-time PCR, werd een standaard ontwikkeld. Deze standaard werd in de test gebruikt (beschreven in Hfd 8) om gelijktijdig DNA en RNA te kwantificeren. Met deze kwantificering was het mogelijk om de groeifase van bacteriën in klinisch materiaal te bepalen. Tevens kon het effect van antibiotica op de groei van bacteriën gevolgd worden . Deze methode is geschikt voor het bepalen van de effectiviteit van antimicrobiële therapie in patiënten. Dit kan leiden tot het optimaliseren, aanpassen en verbeteren van behandelingsmethoden.

In de Sanquin bloedbank regio Noordwest is *Propionibacterium acnes* verantwoordelijk voor de meeste gevallen van bacteriële contaminatie van PCs. Verondersteld wordt dat dit microorganisme afkomstig is van de huidflora van de donor. Verschillende maatregelen waaronder het verwijderen van de eerste 10 mL volbloed, en het verbeteren van desinfectie methoden van de arm van de donor hebben wel geleid tot een verlaging van het totaal aantal contaminaties maar niet tot een vermindering van contaminatie met *Propionibacterium* species. Een studie om de bron en de aard van de contaminanten te identificeren werd in Hfd 7 uitgevoerd. M.b.v amplified fragment length polymorphism (AFLP) werden *Propionibacterium* isolaten uit PCs en bijbehorende RBCs geanalyseerd. De isolaten konden in drie groepen ingedeeld worden. Groep 1 van de isolaten behoorde tot flora van het oppervlak van de huid en toonden complete homologie met *P. acnes*. Isolaten van groep 2 en 3 verschilden op moleculair niveau van groep 1 en vielen waarschijnlijk onder een ander (sub)species. Wat de bron van deze contaminanten was bleef onbeantwoord. Vervolgstudies zijn nodig om andere bacteriën die geassocieerd zijn met contaminatie van bloedproducten te typeren. Inzicht krijgen in het mechanisme van contaminatie kan resulteren in het nemen van adequate maatregelen om contaminatie te voorkomen of in ieder geval te verminderen.

Résumé

Résumé

Les plaquettes ou thrombocytes sont des cellules sanguines anuclées produites dans la moelle osseuse par la fragmentation des mégacaryocytes. Elles sont responsables de la coagulation du sang. Quand il y a une lésion dans un vaisseau sanguin, les plaquettes adhèrent entere elle et au site de lésion, formant ainsi un « clou plaquettaire », suivie après par la formation d'un caillot sanguin. Ce dernier est indisponsable pour l'arrêt de saignement.

Les plaquettes sont utilisées pour la transfusion des patients souffrant de thrombopénie ou de troubles de la fonction plaquettaire. Elles sont également administrées en cas d'augmentation du risque hémorragique pendant certaines interventions chirurgicales lourdes.

Les plaquettes sont préparées à partir de sang total par centrifugation et séparation des composants sanguins. Le don de sang total prélevé au donneur est soumis à une centrifugation rapide, aboutissant à la séparation d'un concentré de globules rouges (CGR), d'une unité de plasma et entre les deux d'une couche leuco-plaquettaire (buffy coat). Pour une dose thérapeutique adulte les couches leuco-plaquettaires provenant de cinq dons de sang sont melangées avec une unité de plasma. Ce mélange subissait ensuite une seconde centrifugation (douce) pour obtenir enfin un concentré plaquettaire (CP).

Le concentré de plaquettes est stocké entre 20 et 24^{o}C sous agitation lente et permanente pendant 5 jours jusqu' à 7 jours si un système de dépistage de CP contaminés est implementé. Ces conditions de stockage sont propices au proliferation des bactéries (lorsqu'elles sont présentes).

La contamination bactérienne des CP constitue aujourd'hui le risque infectieux le plus important de la transfusion sanguine. Pour prévenir, ou du moins réduire, ce risque plusieurs méthodes ont été mises en oeuvre pour identifier les CP contaminés.

Aux Pays-Bas le système de culutre automatisée (automate: BacT/Alert) est utilisé depuis novembre 2001 pour dépsiter les CP contaminés. Après la prépartion des CP, un échantillon (5-10 ml) est inoculé dans les deux flacons de culture aéro- et anérobie de l'automate. Les cultures sont poursuivies 7 jours. Quand la survenue d'une culture positive est signalée le CP et tous les composants sanguins homologues (issus des mêmes dons) sont mises en quarantaine et envoyés pour confirmer et identifier les bactéries présentes. La faisabilité et la capacité de cette méthode pour la révélation des CP contaminés ont été demontrés. Or, le délai

long pour obtenir des résultats (24 -48 heurs) constitue l'inconvénient principal de la culture automatisée: comme la distribution des CP repose sur le critère du « negative to date » des résultats positifs peuvent survenir après l'utilisation des CP. Ce qui représente un risque vital pour le patient transfusé.

L'objectif principal de cette thèse était de développer une nouvelle méthode de détection qui permet le dépistage rapide des CP contaminés et qui évite les limitations de la culture automatisées.

Une méthode moléculaire faisant appel à la technologie de la réaction de polymérisation en chaîne (PCR) en temps réel a été développée. Cette méthode repose sur la détection de l'ADNr 16S de bactéries par PCR quantitative en temps réel. Les étapes de développement et d'optimisation sont décrites aux chapitres 2, 3 et 4. les résultats démontrent la révélation de détecter 1 UFC/PCR (50UFC/mL) par l'ADNr 16S PCR. Les résultats sont disponibles en 4 heures. Pour valider la méthode l'analyse sur 2146 CP a été realisée (chapitre 5). Cette validation a été menée en conjonction avec la culture automatisée. La performance de ce test en ce qui concerne la sensibilité et la spécificité est comparable à celle de la culture automatisée. L'avantage de la méthode est sa rapidité: on peut obtenir les résultats en 4 heures ce qui permet d'utiltiser le test immédiatemet avant la distribution ou la transfusion des CP.

Pour déterminer le moment le plus convenable pour le prélèvement de l'échantillon à analyser, une étude (chapitre 6) était réalisée sur des CP volontairement contaminés avec des inoculums de 5 souches, afin d'obtenir une concentratin finale égale à 1, 10 et 100 UFC/mL. La culture automatisée était utilisée comme référence. Après 24 h de stockage d'incubation le PCR test a révélé la détection de toutes les souches dans 100% des cas en 4 heures. Cependant, la révelation par l'automate BacT/Alert nécessitait une incubation de 20.1 h (au minimum). Basé sur les résultats de cette étude il est donc préférable de prélever un échantillon pour l'analyse par le PCR un jour après la préparation des CP.

La méthode de PCR quantitative ne permet pas de différencier entre l'ADN de bactéries viable et l'ADN de bactéries non viable. Pour distinguer entre les deux, une méthode basée sur la Transcription Inverse RT-PCR quantitative en temps réel a été developpée (chapitre 8). Cette méthode pourrait aussi être utilisée pour déterminer la sensibilité aux antibiotiques. En effect, la faisabilité de ce test reste a être démontrée.

Propionibacterium acnes est fréquemment impliquée dans la contamination des CP. Cette bactérie est rarement considérée comme pathogène. Elle est vu comme un contaminant exogène de produits sanguins. Cette contamination pourrait être introduite au moment de prélèvement de sang total, particulièremnt lors de désinfection de la peau, à l'étape de la préparation des CP ou au moment de l'ensemencement des flacons de cultures de système BacT/Alert.

Lors ce project, une étude a été menée pour identifier l'origine de la contamination des CP par *P. acnes*. Des souches de *Propinibacterium* provenant des CP et des CGR homologues (issus des mêmes dons) ont été alors analysées par la technique de l'AFLP (Polymorphisme de Longueur de Fragments Amplifiés). Sur la base des donnnées de l'AFLP (chapitre 7) les souches se retrouvent dans 3 groupes différents : le premier groupe incluant la plupart des souches de *P. acnes* présentes à la surface de la peau. Le deuxième et le troisième groupe contenant des souches sont différents de premier groupe au niveau moléculair ce qui suggère que ses souches sont possiblement d'origine différente. Afin de mieux comprendre le mécanisme de contamination bactérienne des CP, d'autres études seront nécessaires pour mettre en évidence la diversité génétique des bactéries liées à la contamination de produits sanguins labiles. Une meilleure connaissance et caractérisation moléculaire des espèces impliquées dans la contamination pourrait améliorer la prévention du risque, au du moins la réduction de contamination bactérienne des CP.

Dankwoord

Dankwoord

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